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14. ABSTRACT
This project is to use a unique mouse model to study the interaction of reproductive factors and genetic mutations in the development of ovarian cancer. Ovarian cancer often develops in women of peri-menopausal age. We found that the germ cell deficient Wv mice mimics postmenopausal biology and develop benign ovarian tumors. We plan to test the hypothesis that a synergy exists between oncogenic mutations such as p53, pten, or p27kip1 and postmenopausal biology in ovarian cancer development. In the first year of the project, we completed Aim 1, the study of ovarian tumor phenotypes in mice of compound genotypes. We found that crossing of Wv mice into mutant p53, pten, or p27 background did not lead to a malignant tumor phenotype. Instead, the mutants rescue ovarian germ cells, a very interesting finding. The ovarian surface epithelia in these compound mutant mice develop unique lesions with peculiar morphology, which are undergoing analysis as planned in Aim 2. In future study, we plan to use flox-p53 mutant mice to create mutation only in ovarian surface epithelial but not in germ cells (Aim 3). In sum, the project progresses as planned. We have layered the basis and are posed to further advance.

15. SUBJECT TERMS
Ovarian cancer, mouse model, menopause, preneoplastic lesions, reproductive factors.

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1. Introduction

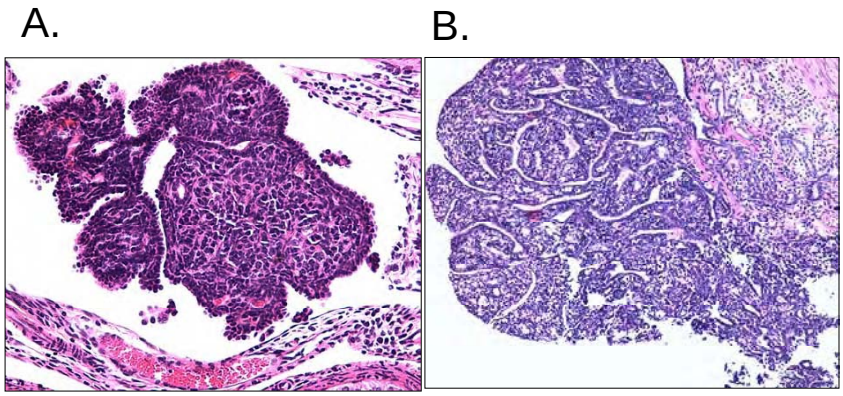
This project is to use a unique Wv mouse model to study the interaction of reproductive factors and genetic mutations in the development of ovarian cancer. Ovarian cancer often develops in women of peri-menopausal age, when ovulation ceases but gonadotropin levels are increased. We found that the germ cell deficient Wv mice mimics postmenopausal biology and develop benign ovarian tumors. We plan to test the hypothesis that the synergy between oncogenic mutations and postmenopausal biology can be revealed by combining the germ-cell deficient phenotype of the Wv/Wv mice and genetic alterations such as p53, pten, or p27kip1, which are found in human ovarian cancer.

2. Body: Research Progress

Previously in the first year of the project, we completed **Aim 1**/*Task 1., t*o determine the optimal genetic changes that synergize with the Wv genotype for the development of malignant ovarian tumors in mice (Months 1-12). We have found that deletion of p53, pten, or p27 in Wv/Wv background each produced unique impacts on the ovarian tumor phenotypes. The details were described in the last report and will not be repeated here. We are still working on various supporting experiments to study the mechanism so that we can publish these interesting findings

In the current or second year, we have selected Wv/Wv;p27 (+/-) and Wv/Wv;p27 (-/-) genotypes for further characterization. Both Wv/Wv;p27 (+/-) and Wv/Wv;p27 (-/-) mice produce large ovarian tumors (**FIGURE 1**). Thus, Wv/Wv;p27 (+/-) or (-/-) represents a new mouse model for ovarian tumors.

FIGURE 1. Ovarian morphology of Wv/Wv:p27 (-/-) and (+/-) ovaries. H&E images of representative ovaries of a 6-month-old Wv/Wv:p27 (-/-) (**A**) and 11-month-old Wv/Wv:p27 (+/-) (**B**) mice. H&E staining shows the unique morphology of the ovarian tumors, which resemble the histology of human ovarian tumors.



1239D p27 (-/-); Wv/Wv, 6 months 1577 p27 (+/-);Wv/Wv, 11 months

Because p53 mutantion is a frequent event in human ovarian cancer, we continue to be interested in producing p53 mutation in the epithelial cells of the Wv/Wv mice. For this effort, we crossed Wv mice with flox-p53 mice to produce Wv/Wv;p53 flox/flox mice. These mice were then be injected with adenoviral cre expressing construct to delete p53 in ovarian surface epithelial cells. To determine the efficiency of adenoviral infection and cre expression, we used beta-galactosidase expressing adenoviral vector as indicator. In control ovaries, we found that

injection of adenoviral-beta-galactosidase led to expression exclusively in the ovarian surface epithelium (**FIGURE 2**). In 3-month-old Wv/Wv mice, the expression of beta-galactosidase was found mainly on the surface, and there was also some internal epithelial cells showing positive staining (**FIGURE 3**). These studies demonstrated that we will be able to efficiently expressing cre in ovarian surface epithelial cells both in control and Wv/Wv mice.

FIGURE 2. Beta-galactosidase staining of wildtype ovaries to determine transfection efficiency. Mice of 3-month-old wildtype were injected with solvent control (**A**) or 10^8 pfu adenovirus expressing beta-gal (**B**) into the ovarian bursa. One week after injection, the ovaries were harvested, sectioned, and stained for beta-galactosidase activity. The beta-gal activity is detected mainly in surface epithelium as shown by an enlarged view (**C**). It is estimated that 70% of the ovarian surface epithelial cells were infected with adenovirus.

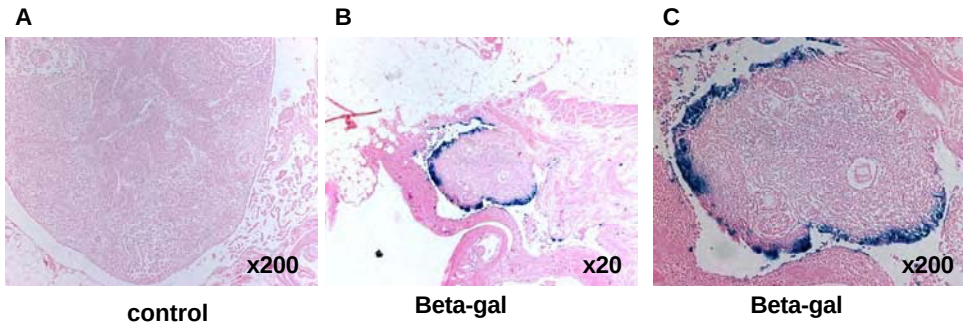
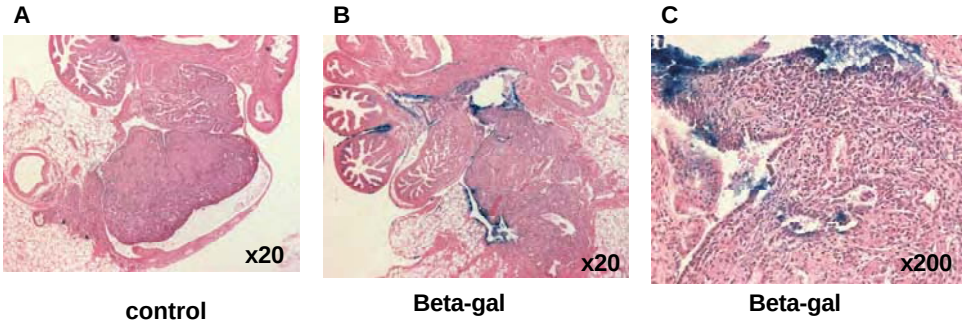


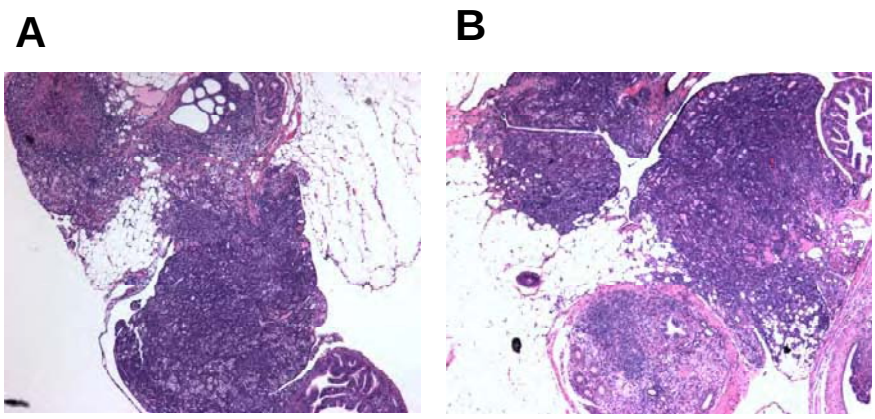
FIGURE 3. Beta-galactosidase staining of Wv/Wv ovary to determine transfection efficiency. Wv/Wv Mice of 3-month-old were injected with solvent control (**A**) or 10^8 pfu adenovirus expressing beta-gal (**B**) into the ovarian bursa. One week after injection, the ovaries were harvested, sectioned, and stained for beta-galactosidase activity. The beta-gal activity is detected mainly in surface epithelium as shown by an enlarged view (**C**). It is estimated that 50% of the ovarian surface epithelial cells were infected with adenovirus. Most of the beta-gal positive epithelial cells are peripheral.



We have also started experiments to inject adeno-cre into the ovaries of Wv/Wv;p53 flox/flox mice. Initial observation suggests that expression of cre produced a drastic impact on the ovarian tumor morphology and sizes, as an example showing in **FIGURE 4**. It appears p53 deletion enhances the expansion of the ovarian tumor. The result is preliminary but is very promising. We are in the process of injecting a larger number of Wv/Wv;p53 flox/flox mice

with adeno-cre, and we also plan to observe the impact on the ovarian tumor phenotype in older (12 months) animals.

FIGURE 4. Ovarian morphology of Wv/Wv:p53 flox/flox (-/-) mice injected with Adeno-beta-gal. Mice of Wv/Wv:p53 (flox/flox) genotype were injected with adenovirus expressing cre at 3 month of age. Five months following injection of cre to delete p53 gene, the ovaries were harvested for H&E staining of right (**A**) and left (**B**) ovaries. The tumors are larger and showing more malignant morphology. The detail analysis and immunological staining of the tumors are ongoing.



Thus, in summary, the pilot study of task 1 or **Aim 1.** (To compare tumor phenotypes of Wv/Wv:p53 (-/-), Wv/Wv:p27 (-/-), and Wv/Wv:pten (+/-)) is completed in year one. We chose to study the wv/wv;p27 (-/-) and Wv/Wv:p53(flox/flox) models in details in *Task 2* (To characterize the selected ovarian tumor mouse model in detail (Months 13-36)). The experiments in task *2* continue to progress into the 3rd/last year as planned. We expect to obtain results and conclude the project by the end of the third year.

Task 3 is "To analyze the alterations in signaling pathways in ovarian tumors and derived cell cultures from the mice (Months 30-36)". The work will be started in year 3, after the establishment and characterization of ovarian cancer mouse model. No experiments have been done in this task yet. Currently, we are ready to initiate the analysis of both wv/wv;p27 (-/-) and Wv/Wv:p53(flox/flox) models as planned for **Aim 3**/Task 3 in the 3rd year of the project.

The future experiments will be carried out essentially as that were planned in the original application. The timeline and tasks in the Statement of Work have been well followed.

Therefore, we report that the project is progress well as planned in the second year. We have completed Task 1, made very good progress on Task 2, and we are ready to engage Task 3 in the coming year. Preliminary results suggest that we will be able to complete our goal of producing a mouse model reflecting the etiology of postmenopausal ovarian cancer risk. We are confident to obtain conclusive results in the coming years.

3. Key Research Accomplishments

- Further verify the relevance of the Wv mouse model to human menopausal biology.

- Verifying the influence of reproductive aging/menopause on human ovarian morphological changes. This finding provides additional support for relevance and rationale to study the Wv mice as models to investigate menopausal physiology on ovarian epithelial remodeling and cancer risk.

- We made an unexpected but very interesting finding that the additional mutation of p53 in the Wv mice rescued ovarian tumor phenotype and preserved germ cells. This finding suggests p53 is very important for the survival and lifespan of ovarian germ cells and follicles. Also, the finding suggests that the depletion of follicles is key for the ovarian tumor phenotype.

- We found that deletion of p27Kip1 enhance the growth of the ovarian tumors. Thus, Wv/Wv; p27 (-/-) genotype is a new mouse model for ovarian tumors.

- Preliminary study showed that Wv/Wv:p53 flox/flox model is technically achievable by our laboratory. The initial result suggests that we will be able to induce ovarian tumors more closely mimicking human cancer using adeno-cre injection in the Wv/Wv:p53 flox/flox mice.

4. Reportable Outcomes

(1) Kathy Q. Cai, Jennifer Smedberg, Elizabeth R. Smith, Andrew K. Godwin, and Xiang-Xi Xu, 2007 in preparasion. We showed that the Wv mice in combination with p27 is a new mouse model for postmenopausal biology, ovarian aging, and ovarian cancer risk. 2007; in preparation.

(2) Yang et al., Am. J. Pathology, 2007 in press: We showed that the Wv mice are excellent models for human menopausal biology. This result is published.

(3) Cai, Yang, Smith et al., 2007 in preparasion: We found that p53, pten, and p27kip1 genes have critical impacts on the survival of ovarian germ cells/follicle. We are doing additional experiments to characterize the mechanism further and also to obtain additional cases to determine the statistical significance. These results will be prepared for publication in the near future.

5. Conclusions:

The experimental results are supportive of the hypothesis that addition of genetic mutations may convert the benign ovaian tumors in Wv mice into more malignant tumors.

To add p53 mutation, we need to delete p53 specifically in ovarian surface epithelial cells but not in germ cells tin order to create a model of malignant ovarian tumor based on the Wv mice. In the current study, we introduce mutation only in ovarian surface epithelial cells to avoid the rescuing activity in germ cells. We have crossed Wv mice into flox-p53 mutant mice. We have introduced cre recombinase into ovarian surface epithelial cells in the mice by injecting adeno-cre into ovary to delete p53 only in surface epithelial cells but not in germ cells. This

approach was considered in the original application as an alternative approach. Thus, the future experiments will be carried out essentially as that were planned in the original application.

In sum, the project is progress well as planned. We have layered the basis in the first 2 years of the project, and we hope to obtain conclusive results for the aims and questions proposed in the coming 3rd/last year.

6. References

Cai, K.Q, Yang W.L., Capo-chichi C.D., Vanderveer, L., Wu H., Godwin A.K., and Xu X.X. Prominent Expression of metalloproteinases in early stages of ovarian tumorigenesis. Mol Carcinog. 2007 Feb;46(2):130-43.

Yang, W.L., Klein-Szanto, A., Hamilton, T.C., and Xu, X.X. A reduction of Cox-2 gene dosage counters the menopausal ovarian morphological aging and tumor phenotype in Wv mice. Am. J. Pathol. 2006; Am J Pathol. 2007 Apr;170(4):1325-36.

Wan-Lin Yang, Kathy Q. Cai, Jennifer Smedberg, Elizabeth R. Smith, Andres Klein-Szanto, Thomas C. Hamilton and Xiang-Xi Xu. Life-span prolongation of follicles and germ cells in Wv mice following reduction of cyclooxygenase activities. 2007; in preparation.

Kathy Q. Cai, Jennifer Smedberg, Elizabeth R. Smith, Andrew K. Godwin, and Xiang-Xi Xu. A mouse model for postmenopausal biology and ovarian aging. 2007; in preparation.

7. Appendices

Cai, K.Q, Yang W.L., Capo-chichi C.D., Vanderveer, L., Wu H., Godwin A.K., and Xu X.X. Prominent Expression of metalloproteinases in early stages of ovarian tumorigenesis. Mol Carcinog. 2007 Feb;46(2):130-43.

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Tumorigenesis and Neoplastic Progression

A Reduction of Cyclooxygenase 2 Gene Dosage Counters the Ovarian Morphological Aging and Tumor Phenotype in Wv Mice

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Menopausal ovaries undergo morphological changes, known as ovarian aging, which are implicated in the high incidence of ovarian cancer occurring during the perimenopausal and immediate postmenopausal periods. The germ cell-deficient Wv mice recapitulate these postmenopausal alterations in ovarian morphology and develop tubular adenomas. We demonstrate that a reduction of cyclooxygenase 2 gene dosage rescued the ovarian aging phenotype of the Wv mice, whereas homozygous deletion was accompanied by a compensatory increase in ovarian cyclooxygenase 1 expression and prostaglandin E_2 synthesis. Cyclooxygenase inhibitors also reduced the tumor phenotype in a preliminary study. These findings suggest that increased cyclooxygenase activity contributes to the preneoplastic morphological changes of the ovarian surface epithelium, which can be reversed by a reduction of gene dosage achieved by either genetic or pharmacological approaches. ***(Am J Pathol 2007, 170:1325–1336; DOI: 10.2353/ajpath.2007.060769)***

Menopause is defined as the permanent cessation of menstruation resulting from depletion of germ cells and loss of ovarian follicular activity, and it is accepted to be a by-product of modern health advances and the extension of lifespan that occurred in the last century.[1] By the end of the reproductive age, germ cells and follicles are depleted from the ovaries, and the ovulatory cycle ceases, resulting in menopause. The perimenopausal period commences when the first features of menopause begin until at least 1 year after the final menstrual period, generally lasting an average of 5 years. In humans, the transition to menopause is a set of gradual changes, in which ovarian function, reproductive capacity, and hormonal status are altered long before menses stops completely. Menopause generally occurs between 45 to 55 years of age, and the symptoms vary among women.

After menopause, estrogen levels fall, but the gonadotropins including luteinizing hormone and follicle-stimulating hormone (FSH) are elevated and often even higher than before menopause.[1] The incidence of ovarian cancer is highest in the perimenopausal period, which supports the gonadotropin stimulation theory of ovarian cancer etiology. Among the physiological changes associated with menopause, the ovarian tissues undergo morphological transformation, known as ovarian aging, and this is implicated in the high incidence of ovarian cancer that occurs during the perimenopausal and immediate postmenopausal periods.[1–4] One feature associated with ovarian aging is the accumulation of ovarian morphological changes such as deep invaginations, surface papillomatosis, and inclusion cysts (Supplemental Figure 1, see *http://ajp.amjpathol.org*), which are thought by some to be the histological precursors of ovarian cancer.[3,4]

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The phenomenon of menopause is not restricted to human females but also occurs in laboratory rats and mice that exhibit postreproductive lifespan preceded by a period of gradual reproductive decline. A naturally occurring mutant, white spotting variant (Wv) mice harbor a point mutation in the kinase domain of the c-kit gene, resulting in developmental defects in germ cells, pigment-forming cells, red blood cells, and mast cells in homozygous mutant mice.[5–8] The Wv/Wv mice have a similar lifespan as wild type, are sterile, white coated with black eyes, and predisposed to ovarian neoplasms.[9] The Wv/Wv homozygous mice contain less than 1% of the normal number of oocytes at birth, and the remaining oocytes are depleted by ~8 weeks of age.[9] Consequently, ovulation ceases, and an increase in pituitary gonadotropins follows because of the lack of feedback inhibition that is normally mediated through progesterone released from the corpora lutea.[10] The females recapitulate and exaggerate the postmenopausal alterations in ovarian morphology and develop ovarian tubular adenomas.[9] Elevated levels of gonadotropins are believed to be the causative factor of the ovarian neoplasm.[11,12] The ovarian lesions in the Wv mice are known as complex tubular adenomas.[9] These ovarian tumors are generally benign and seldom develop malignant features. Nevertheless, the causative factors, the depletion of germ cells, and subsequent increase in gonadotropins, mimic the condition of perimenopausal women, in which gonadotropin levels are elevated and the risk for ovarian cancer is highest.[13] In addition, the ovarian lesions of the Wv/Wv mice have particular resemblance to morphological changes found in ovaries of women with an increased risk for ovarian cancer. These changes include surface papillomatosis, deep invaginations, and cystadenomas and are considered preneoplastic lesions. Thus, the Wv/Wv mice may constitute a model to investigate how ovulation and gonadotropin stimulation during postmenopause act as etiological factors in ovarian cancer development.

Cyclooxygenase (Cox)-2, encoded by the prostaglandin synthase 2 (*ptgs2*) gene, is a key downstream component of gonadotropin-stimulated signaling in ovulation, and Cox-2 knockout mice are anovulatory.[14–16] The role of Cox-2 in ovulatory rupture of the ovarian surface is similar to an inflammatory process.[17] Cox-2 is often overexpressed in human cancers,[18,19] and suppression of Cox-2 by either genetic or pharmacological approaches has been shown to reduce colon tumor development in mouse models[20–23] and in humans.[24] Inhibition of Cox enzymes by nonsteroidal anti-inflammatory drugs also seems to reduce ovarian cancer risk,[25–27] and a possible mechanism is that inhibition of Cox-2 may reduce the cancer-promoting activity of the inflammation-like ovulatory processes that are stimulated by gonadotropins.[28] In this study, we investigated the role of Cox-2 in the development of ovarian tumors in the germ cell-deficient Wv mice, which model postmenopausal ovarian biology.

Materials and Methods

Generation and Genotyping of Wv:Cox-2 Mutant Mice

An inbreeding colony was established by crossing Wv/+ and Cox-2 (+/−) mice in the C57BL/6J background (Jackson Laboratory, Bar Harbor, ME). Littermates with Wv/+:Cox-2 (+/−) genotype were further intercrossed to generate mutant and control mice for analysis. The Wv genotypes of the resulting progeny were identified by the coat color: white, gray with ventral/dorsal spots, or black represents Wv/Wv, Wv/+, or Wv (+/+), respectively. Cox-2 genotypes were verified by polymerase chain reaction (PCR) analysis using the genomic DNA isolated from mouse tails with the following primers[29]: Cox-2 forward: 5′-GCCCTGAATGAACTGCAGGACG-3′ and reverse: 5′-ACCTCTGCGATGCTCTTCC-3′; Neo forward: 5′-GCCCTGAATGAACTGCAGGACG-3′ and reverse: 5′-CACGGGTAGCCAACGCTATGTC-3′. PCR reactions were set up in a 25-μl total volume with a final concentration of 2.5 U of platinum *Taq*DNA polymerase (Invitrogen, Carlsbad, CA), 1× PCR buffer (Invitrogen), 1.5 mmol/L $MgCl_2$, 0.2 μmol/L primers, 0.2 mmol/L dNTP mixtures (Promega, Madison, WI), and 15 to 20 ng genomic DNA. Cycling conditions were followed as that described by the Jackson Laboratory. In brief, the PCR reactions were performed with 35 cycles consisting of melting at 94°C for 30 seconds, annealing at 66°C for 1 minute, and extension at 72°C for 1 minute. At the end of the PCR reactions, the mixtures were kept for an additional 2 minutes at 72°C and stored at 10°C until analysis. PCR products were resolved by electrophoresis on a 2% agarose gel containing ethidium bromide. The presence of wild-type and neo alleles corresponds to 857-bp and 500-bp products, respectively. Approximately 30 to 40% of homozygous Cox-2 mutant mice were lost during the preweaning stage,[29] and only limited numbers (28 females were obtained and analyzed so far) of mice with the Wv/Wv:Cox-2 (−/−) genotypes were obtained.

Analysis and Quantitation of Tumor Phenotype

The ovaries of 4- to 5-month-old mice with Wv/Wv, Wv/Wv:Cox-2 (+/−), or Wv/Wv:Cox-2 (−/−) genotypes were harvested for histological analysis of ovarian tumor phenotype. The largest cross-section of an ovary was stained with cytokeratin-8 to identify epithelial components, and a digital image was recorded. The degree of tumor phenotype was defined as the percentage of the ovary penetrated by the cytokeratin-8-positive epithelial tubular structure, which was quantitatively calculated by laying a 20 × 20 grid over the ovarian image to divide the ovary into 200 to 300 squares. Grids positive and negative for internal epithelial lesions were counted independently by two noninvolved persons (student assistants) and used to calculate the percentage of ovaries infiltrated by the tubular adenomas.

Preparation of Ovarian Lysate and Western Blotting

Mouse ovaries were snap-frozen on surgical removal and maintained at −80°C until the tissues were used for protein analysis. To prepare ovarian lysates, frozen tissues were homogenized in radioimmunoprecipitation assay (RIPA) buffer (150 mmol/L NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 10 mmol/L Tris, pH 7.2, 100 μmol/L sodium orthovanadate, and 50 mmol/L NaF) containing protease inhibitors (Boehringer Mannheim, Indianapolis, IN) using a Mini-BeadBeater (BioSpec Products, Bartlesville, OK) at 5000 rpm, 20-second interval for a total of 4 minutes. The homogenate was centrifuged at 10,000 × *g* for 10 minutes to remove the particulate material. The protein concentration in the supernatant was measured using the DC protein assay (Bio-Rad, Hercules, CA).

For Western blotting, an aliquot of the total ovarian lysate was separated by electrophoresis on a 4 to 12% gradient gel and electrotransferred onto a polyvinylidene difluoride membrane. Membranes were incubated with antibodies against either Cox-1 or Cox-2 (Cayman Chemicals, Ann Arbor, MI), or β-actin (Sigma, St. Louis, MO) followed by horseradish peroxidase-labeled secondary antibodies (Sigma). The signals were revealed using a chemiluminescence detection system (Pierce, Rockford, IL). Ovarian lysate from Cox-1 (−/−) mice (obtained from Dr. Robert Langenbach, Laboratory of Experimental Carcinogenesis and Mutagenesis, National Institute of Environmental Health Sciences, Research Triangle Park, NC) was used to confirm the specificity of Cox-1 antibodies.

Immunohistochemistry

Ovaries were fixed in buffered formalin and embedded in paraffin. The paraffin blocks were cut into 5-μm-thick sections that were placed on positively charged slides. The sections were dewaxed in xylene and hydrated through graded ethanol. Heat-induced antigen retrieval was then performed in 10 mmol/L sodium citrate (pH 6.0) in a microwave initially at high-power setting (no. 10) for 2 minutes followed by low-power setting (no. 2) for 10 minutes. The endogenous peroxidase activity was blocked by immersing the slides in 3% H_2O_2 in methanol for 15 minutes. After 30 minutes of incubation with blocking serum, slides were incubated with rat monoclonal anti-Troma-1/cytokeratin-8 antibodies (Developmental Studies Hybridoma Bank from The University of Iowa, Ames, IA) at 1:600 dilution, Cox-2 rabbit polyclonal antibodies (Cayman Chemical) at 1:600 dilution, Cox-1 rabbit polyclonal antibodies (Cayman Chemical) at 1:500 dilution, or F4/80 rat polyclonal antibodies (Serotec Ltd., Kidlington, Oxford, UK) at 1:200 dilution at 4°C overnight followed by incubating with goat anti-rat horseradish peroxidase-labeled secondary antibodies (BD Pharmingen, Franklin Lakes, NJ) at 1:100 dilution for cytokeratin-8, anti-rabbit labeled polymer horseradish peroxidase (DAKO, Carpinteria, CA) at 1:100 dilution, or biotinylated anti-rat at 1:200 dilution for F4/80 for 35 minutes at room temperature. Diaminobenzidine was used as the chromogen for the immunoperoxidase reaction. The slides were counterstained with hematoxylin and mounted in 50:50 xylene/Permount.

Prostaglandin E_2 Assay

The prostaglandin E2 (PGE_2) level in the ovarian lysates was measured using an enzyme-immunoassay kit (Cayman Chemical). This assay is based on the competition between PGE_2 and a PGE_2 acetylcholinesterase conjugate (PGE_2 tracer) for a limited amount of PGE_2 monoclonal antibodies. This antibody-PGE_2 complex binds to goat polyclonal anti-mouse IgG that has been attached to the plate. In brief, ovarian lysates together with enzyme-immunoassay buffer, PGE_2 tracer, and antibody were added into the plate. The mixtures were incubated at 4°C overnight. The plates were washed to remove the unbound reagents and developed with the Ellman's reagent (substrate), and the absorption was measured at 412 nm. The amount of PGE_2 in the sample was determined from a standard curve.

FSH Assay

At the time of sacrifice, 100 to 200 μl of sera were collected from each animal and stored at −80°C until testing. The FSH level in the serum was measured by radioimmunoassay through custom service from the National Hormone and Peptide Program, Harbor-UCLA Medical Center (Torrance, CA).

Drug Administration

Four-week-old female Wv/Wv mice, weighing ~14 g, were randomly allocated into three groups: nontreated control ($n = 5$), indomethacin-treated ($n = 7$), and celecoxib (Celebrex)-treated groups ($n = 8$). Celecoxib was administrated through feeding with AIN 76A diet (Dyets Inc., Bethlehem, PA) containing 1500 ppm celecoxib (Fox Chase Cancer Center Pharmacy). Indomethacin was introduced through drinking water (6 mg/ml). During the study, mice were permitted free access to the diet and drinking water. All of the mice were inspected once daily to monitor their general health status. Body weight was measured once per week throughout the experiments. Optimal dosage was estimated to be 100 μg/day for celecoxib and 30 μg/day for indomethacin. Dosages double these amounts resulted in some toxicity such as reduced weight or activity/alertness in the female Wv/Wv mice. Mice were sacrificed at ~4 months of age. Ovarian tissues were collected and subjected to histopathological examination.

Statistical Analysis

Basic and standard analytical procedures were applied to examine the statistical significance of the data. Differences in proportions were evaluated by the χ^2 or the

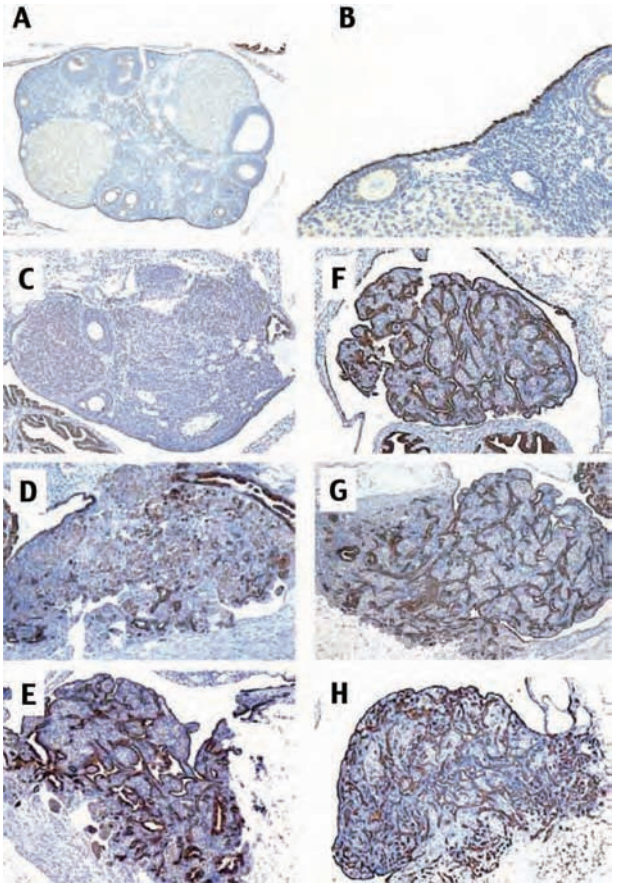

Figure 1. Development of tubular adenomas in Wv/Wv mice. Ovarian morphology was compared between wild-type littermates (**A** and **B**) and Wv/Wv (**C–H**) mice. Cytokeratin-8 (Troma-1) staining was used to highlight epithelial cells. **A:** An ovary from wild-type control at 4 months of age and **B:** at higher magnification. Representative examples of ovaries from Wv/Wv mice at the age of 1 month (**C**), 2 months (**D**), 3 months (**E**), 4 months (**F**), 5 months (**G**), and 9 months (**H**) are shown. At least five mice at each time point were examined. Original magnifications: ×40 (**A**, **C–H**); ×100 (**B**).

Fisher exact test, as appropriate. Student's *t*-test was used to compare the differences in means between two groups. Statistical significance was considered as $P < 0.05$. All P values are two-sided.

Results

Ovarian Surface Epithelia Undergo Morphological Transformation and Tumorigenesis in Wv Mice

We have maintained a colony of Wv mice by inbreeding for the last 3 years. Of the more than 200 mature (3 months or older) Wv/Wv females examined, all exhibited ovarian tubular adenomas, whereas none were observed in the wild-type littermates. The progressive changes in ovarian morphology were documented, and representative examples are shown in Figure 1. As a comparison, the wild-type ovary contains developing follicles of all stages and multiple corpora lutea (Figure 1A). The surface epithelia lining the perimeter of the ovary are the only cells positive for cytokeratin-8, a marker of epithelial cells. Higher magnification reveals the well-organized surface epithelial cells and interstitial stroma cells in the ovarian cortex (Figure 1B). In Wv/Wv mice at 1 month of age, few follicles can be observed in the ovary although the cortex is still enveloped by a smooth layer of cytokeratin-8-positive surface epithelial cells (Figure 1C). We began to observe the presence of tubule-like structures in the ovarian cortex of the Wv/Wv mice by 2 months of age (Figure 1D). By the end of 3 months, the entire ovary was replaced by tumorous lesions. Positive staining for cytokeratin-8 indicates the epithelial origin of the tubular structures (Figure 1E). At 4 and 5 months, the phenotype of tubular adenomas became more complex as shown by the increased penetration and branching of the epithelial tubules (Figure 1, F and G), together with the appearance of scalloping and crowding of epithelial cells into multiple cell layers. We have analyzed the Wv/Wv ovaries for up to 1 year of age when the entire ovaries were completely permeated with the tumor cells. The lesions are somewhat more complex and intense in older mice, but the tumor cells have not further expanded into large masses or acquired malignant features (Figure 1H). Although the ovarian lesions in the Wv mice distribute throughout the ovarian stroma, and are known as stromal tubular adenomas,[9] the contiguous connection to ovarian surface epithelium is evident. This is especially pronounced in cases of early ovarian lesions in younger (7 to 10 weeks) mice when only a few lesions have developed. Obvious surface origination of the epithelial lesions can be observed (Figure 2A, arrow): the tubular structure is contiguous with the monolayer of the surface epithelium (Figure 2B). We conclude that most if not all of the tubular adenomas in Wv/Wv ovaries are derived from ovarian surface epithelial cells. The majority of the lesions either exhibit inclusion cyst-like structures (Figure 2C) or resemble surface deep invaginations/papillomatosis (Figure 2D). Although dysplastic morphology is evident in some epithelial compartments of the tubular adenomas (Figure 2, E and F), the ovarian epithelial tubular structures in the Wv mice are considered benign tumors and the lesions are confined to ovarian tissues and do not become metastatic. Histopathological evaluation also indicated their benign cytological morphologies without evident mitotic figures. These observations are consistent with the notion that the benign epithelial tubular adenomas are caused by the stimulation of the reproductive hormones gonadotropins rather than oncogenic alterations. Indeed, we confirmed that serum FSH, a key gonadotropin, is greatly increased (~10-fold) in female Wv/Wv mice (Figure 2G). The magnitude of FSH increase is very similar to the elevation found in menopausal women.[1] Thus, it seems that the Wv/Wv mouse model closely mimics the changes in ovarian physiology (follicle depletion), endocrine factor (hormonal increase), and ovarian pathology (morphological changes) in menopausal women. In the investigation of potential ovarian mediators of gonadotropins, we found that ovarian

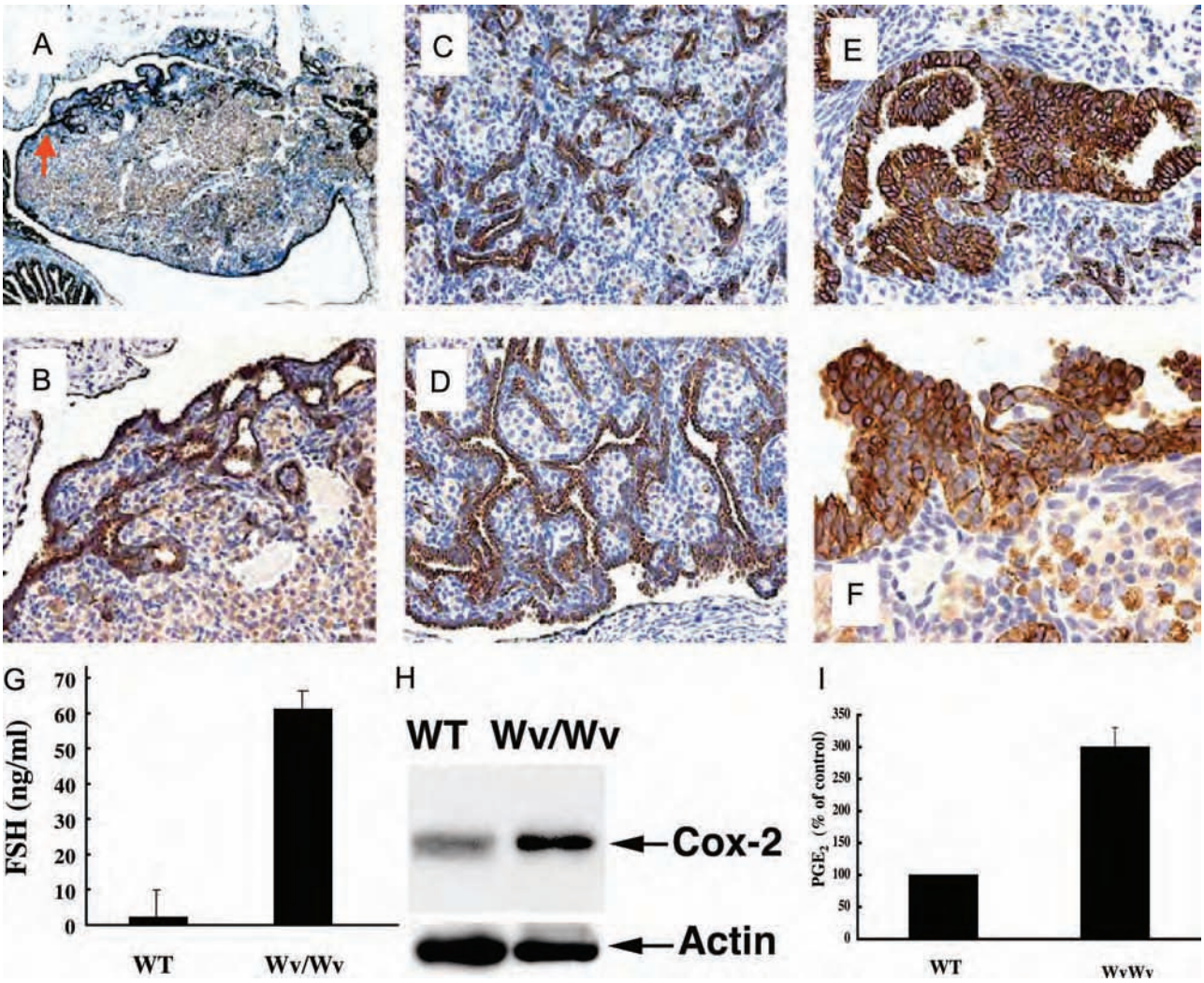

Figure 2. Ovarian morphological and physiological changes in Wv/Wv mice. Ovarian tissues were harvested and subjected to histological analysis. Cytokeratin-8 staining was used as a marker for epithelial cells. **A:** An example of an ovary from a 7-week-old Wv/Wv mouse is shown for the surface epithelial origin of the tubular adenoma structure. The **arrow** indicates the contiguous links between surface epithelium and the tubular epithelial structure, which are stained for cytokeratin-8, and the area is shown at a higher magnification in **B**. **C** and **D:** Two representative examples of tubular adenomas; and **E** and **F:** two examples of dysplastic epithelial cells from ovarian tubular adenomas of 4-month-old Wv/Wv mice. **G:** Serum was collected from four each of wild-type and Wv/Wv 4-month-old female mice to determine FSH levels in triplicate. Averages of triplicate with SDs are shown. Two-sided Student's *t*-test indicates the difference is statistically significant ($P < 0.001$). **H** and **I:** Ovaries ($n = 10$) from five female mice each, of either Wv/Wv or wild-type littermates at 4 months of age, were collected, dissected to remove the surrounding fat tissues, and pooled. The tissues were homogenized in RIPA buffer, and the lysate was used for measurement of Cox-2 protein by Western blot (**H**) and PGE_2 level by EIA (**I**). Actin level was used as a protein loading control in Western blotting. Averages of triplicate with SDs are shown for PGE_2 levels. Two-sided Student's *t*-test indicates the difference is statistically significant ($P < 0.001$). Examples of ovarian morphology are shown by H&E staining in Supplemental Figure 2 (see *http://ajp.amjpathol.org*). Original magnifications: ×40 (**A**); ×200 (**B–F**).

Cox-2 protein levels are dramatically increased in Wv/Wv mice compared with those of control littermates (Figure 2H). Accordingly, the ovarian PGE_2 level is threefold to fivefold higher in Wv/Wv mice (Figure 2I).

Wv Mouse Tumor Phenotype Is Suppressed by a Reduction of Cox-2 Gene Dosage

We investigated whether Cox-2 plays a role in the formation of tubular adenomas in Wv mice in which the elevated gonadotropins likely stimulate Cox-2 expression. Cox-2 deficiency was introduced into the Wv mouse colony by crossing Wv/+ with Cox-2 (+/−) mice, and a new inbred colony was established by crossing Wv/+:Cox-2 (+/−) siblings. Progenies homozygous for Wv and all genotypes of Cox-2, (+/+), (+/−), or (−/−), were examined for ovarian morphology at ~4 months of age (Figure 3), when the tubular adenoma phenotype seems to be fully presented and fairly uniform in the Wv/Wv mice. The ovarian tubular adenoma phenotype in the 70 Wv/Wv:Cox-2 (+/+) mice produced in this colony was essentially identical to that found in more than 200 female mice of the original colony before introduction of the Cox-2 mutation: all of the ovaries exhibited severe complex tubular adenomas that permeated the entire organ. We observed a significant alleviation of ovarian lesions in the Wv/Wv:Cox-2 (+/−) ovaries analyzed (Figure 3, D–F),

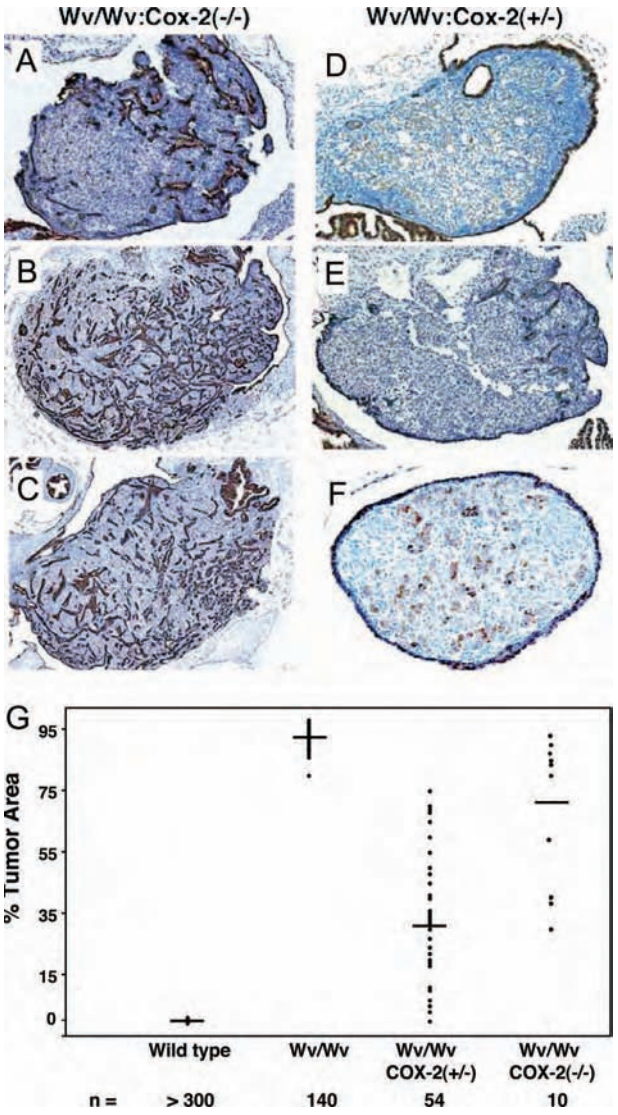

Figure 3. Rescue of Wv/Wv mouse ovarian tubular adenoma phenotype by the Cox-2 knockout. The ovarian morphology from littermates of the Wv and Cox-2 knockout inbred colony was compared. Cytokeratin-8 (Troma-1) staining was used to identify epithelial cells. Representative examples of ovaries from 4.5-month-old mice of Wv/Wv:Cox-2 (−/−) (**A–C**) and Wv/Wv:Cox-2 (+/−) (**D–F**) genotypes are shown. The image shown in **A** is the example of ovary ($n = 10$) from Wv/Wv:Cox-2 (−/−) mice with significant reduction in ovarian lesions. **G:** The degree of tumor involvement of each ovary was estimated and the distribution is plotted. *n* indicates the number of ovaries analyzed. The mean value for ovarian tumor involvement is 0% for wild type, 93% for Wv/Wv, 32% for Wv/Wv:Cox-2 (+/−), and 70% for Wv/Wv:Cox-2 (−/−) genotypes. Student's *t*-tests showed $P < 0.005$ for Wv/Wv versus Wv/Wv:Cox-2 (+/−), and $P < 0.05$ for Wv/Wv versus Wv/Wv:Cox-2 (−/−), indicating a statistically significant difference. The difference between Wv/Wv:Cox-2 (+/−) and Wv/Wv:Cox-2 (−/−) also is statistically significant ($P < 0.001$). Examples of ovarian morphology are shown by H&E staining in Supplemental Figure 3 (see *http://ajp.amjpathol.org*). Original magnifications, ×40.

although the degree to which the tumor phenotype was suppressed varied greatly (Figure 3G). Some ovaries completely lacked epithelial tubular structures inside the cortex (Figure 3, D and F), and some contained only a small number of epithelial tubular structures (Figure 3E). Unlike wild-type ovaries, these Wv/Wv:Cox-2 (+/−) ovaries lacked apparent follicles or corpora lutea. Thus, hemizygous reduction of the Cox-2 gene resulted in a complete (Figure 3, D and F) or partial (Figure 3E) rescue from the epithelial adenoma phenotype. Unexpectedly, of the 10 ovaries from mice of Wv/Wv:Cox-2 (−/−) genotype, only three ovaries exhibited a significant (50% area) reduction in the tubular adenoma phenotype (Figure 3, A and G). All other ovaries showed ovarian morphology indistinguishable from that of Wv/Wv:Cox-2 (+/+) genotype (Figure 3, B and C). The mean value for the area of the ovary covered by lesions is 93% for Wv/Wv, 32% for Wv/Wv:Cox-2 (+/−), and 70% for Wv/Wv:Cox-2 (−/−) genotypes (Figure 3G). Thus, a reduction in Cox-2 gene dosage rescued the ovarian epithelial morphological alteration, but deletion of both copies was less sufficient in reversing the adenoma phenotype in Wv/Wv mice.

Cox-2-Null Deletion Causes a Compensatory Increase in Cox-1 Expression in Ovaries

Measurement of prostaglandin levels in ovaries of these mice showed that Wv/Wv mice exhibited an increase of ovarian PGE_2 (Figure 4A) and Cox-2 (Figure 4B) levels over the wild-type littermates, to 4.2- and 2.1-fold, respectively. The expression of the Cox-1 protein was also elevated over that of wild type (3.5-fold). This increase in the Cox-1 level is unexpected because it is commonly accepted that Cox-1 is a housekeeping gene and generally not regulated.[30] However, Cox-1 expression was previously found increased in human ovarian cancer[31,32] and in ovarian tumors found in a variety of mouse ovarian cancer models.[33]

Ablation of one allele of Cox-2 lowered the amount of PGE_2 produced in the ovaries by 45% (compare Wv/Wv:Cox-2 (+/−) to Wv/Wv) (Figure 4A). The prostaglandin level in Wv/Wv:Cox-2 (−/−) mice was higher than that of Wv/Wv:Cox-2 (+/−) and similar to that of Wv/Wv mice (Figure 4A). The ovarian Cox-1 protein amount in Wv/Wv:Cox-2 (−/−) was determined to be 2.8-fold of those in Wv/Wv or Wv/Wv:Cox-2 (+/−) mice (Figure 4C). Presumably, the Cox-2 homozygous deletion causes a compensatory increase in Cox-1 and total prostaglandin level. The Cox-1 compensation was also observed in non-Wv Cox-2-null mice (Figure 4, D and E). In the ovarian extract, the PGE_2 level was increased 25% in Cox-2 homozygous knockout mice, although ovarian PGE_2 was 20% less in Cox-2 hemizygous mice (Figure 4D). Accordingly, ovarian Cox-1 protein was increased 2.1-fold in Cox-2 (−/−) mice but remained unchanged in Cox-2 (+/−) mice (Figure 4E). The expression of Cox-1 and Cox-2 was analyzed by immunostaining in wild-type and Wv/Wv ovaries (Figure 4F). Weak Cox-1 staining distributed evenly throughout the wild-type ovary, and Cox-2 staining was observed in stromal and follicular cells but not in surface epithelial cells. In Wv/Wv ovaries, both epithelial and stromal cells stained strongly for Cox-1 and Cox-2 (Figure 4F).

We studied the epithelial and stromal compartments to determine whether tumor phenotype and Cox-2 expres-

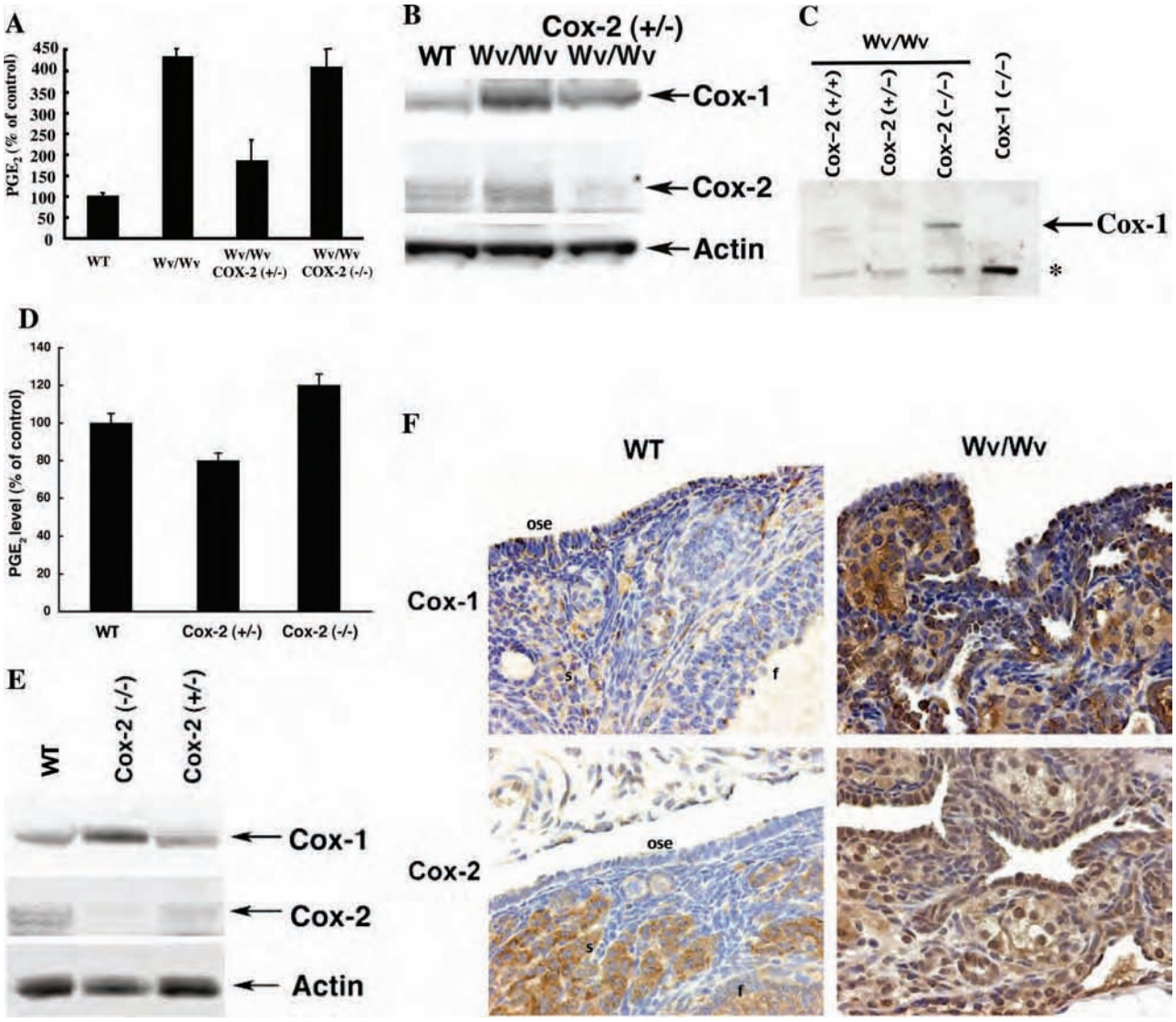

Figure 4. Ovarian PGE_2, Cox-1, and Cox-2 levels in Wv and Cox-2 mutant mice. Ovaries (n = 4 to 10) from female mice, of either Cox-2 (−/−), Cox-2 (+/−), Wv/Wv, Wv/Wv:Cox-2 (+/−), or wild-type littermates at 4 months of age were collected and pooled. The tissues were homogenized in RIPA buffer, and the lysate was used for measurement of PGE_2 level by EIA. Relative values compared with wild type (WT) are reported as the percentage of the wild-type control, which is 3.4 pg/μg protein. **A:** Three experiments were performed to obtain the representative result, and the averages of triplicate with SDs are shown. Two-sided Student's t-test indicates the difference is statistically significant for the comparisons between WT and Wv/Wv ($P < 0.0001$), WT and Wv/Wv:Cox-2 (+/−) ($P < 0.001$), WT and Wv/Wv:Cox-2 (−/−) ($P < 0.0028$), Wv/Wv and Wv/Wv:Cox-2 (+/−) ($P < 0.0001$), and Wv/Wv:Cox-2 (+/−) and Wv/Wv:Cox-2 (−/−) ($P < 0.0002$), but the difference does not reach statistical significance comparing Wv/Wv and Wv/Wv:Cox-2 (−/−) ($P > 0.12$). **B:** Cox-1 and Cox-2 proteins in the ovarian lysate from a pool of 10 ovaries of each genotype were determined by Western blot with actin as loading control. **C:** One of two representative experiments was shown for the determination Cox-1 protein from two ovaries (one mouse) by Western blot. Ovarian lysate from a Cox-1 (−/−) mouse was used for control for the specificity of the anti-Cox-1 antibodies. *Nonspecific protein band that served as a protein loading control. **D** and **E:** Ovaries (n = 10) from five mice each of either Cox-2 (−/−), Cox-2 (+/−), or wild-type (WT) littermates at 4 months of age were collected for PGE_2 assays (**D**) and Western blot analysis for Cox-1 and Cox-2 proteins with actin as a loading control (**E**). Two-sided Student's t-test indicates the differences between the PGE2 levels are statistically significant comparing WT and Cox-2 (+/−) ($P < 0.02$) or WT and Cox-2 (−/−) ($P < 0.04$). **F:** Ovaries from wild-type and Wv/Wv genotypes were stained for Cox-1 or Cox-2. ose, ovarian surface epithelial; s, stroma; f, follicle. Original magnifications, ×200.

sion correlated with inflammation in the ovaries. Infiltration of neutrophils and eosinophilic cells was rarely observed in Wv mouse ovaries and tumors, and no evidence of acute inflammation was found. We also examined macrophage density that associates with chronic inflammation by staining for the F4/80 marker (Figure 5, A and B). F4/80 staining indicated a robust increase in the appearance of macrophages in the tumorous Wv/Wv ovaries over those of the wild-type littermates (Figure 5A). Consistently, the macrophage number was reduced in the recovered ovaries of Wv/Wv:Cox-2 (+/−) genotype but not of the Wv/Wv:Cox-2 (−/−) genotype (Figure 5, A and C). Moreover, in nontumorous wild-type and Cox-2 (−/−) ovaries, macrophages were distributed in the cortex and stromal compartments but were absent in or near epithelial cells. In the ovarian tumors from Wv/Wv mice, macrophages often infiltrated into the epithelia (Figure 5B, red arrow). The relative macrophage density was quantitated as shown in Figure 5C. The correlation of macrophage density with tumor phenotypes suggests that inflammatory reactions promote the tubular adenoma development in Wv ovaries.

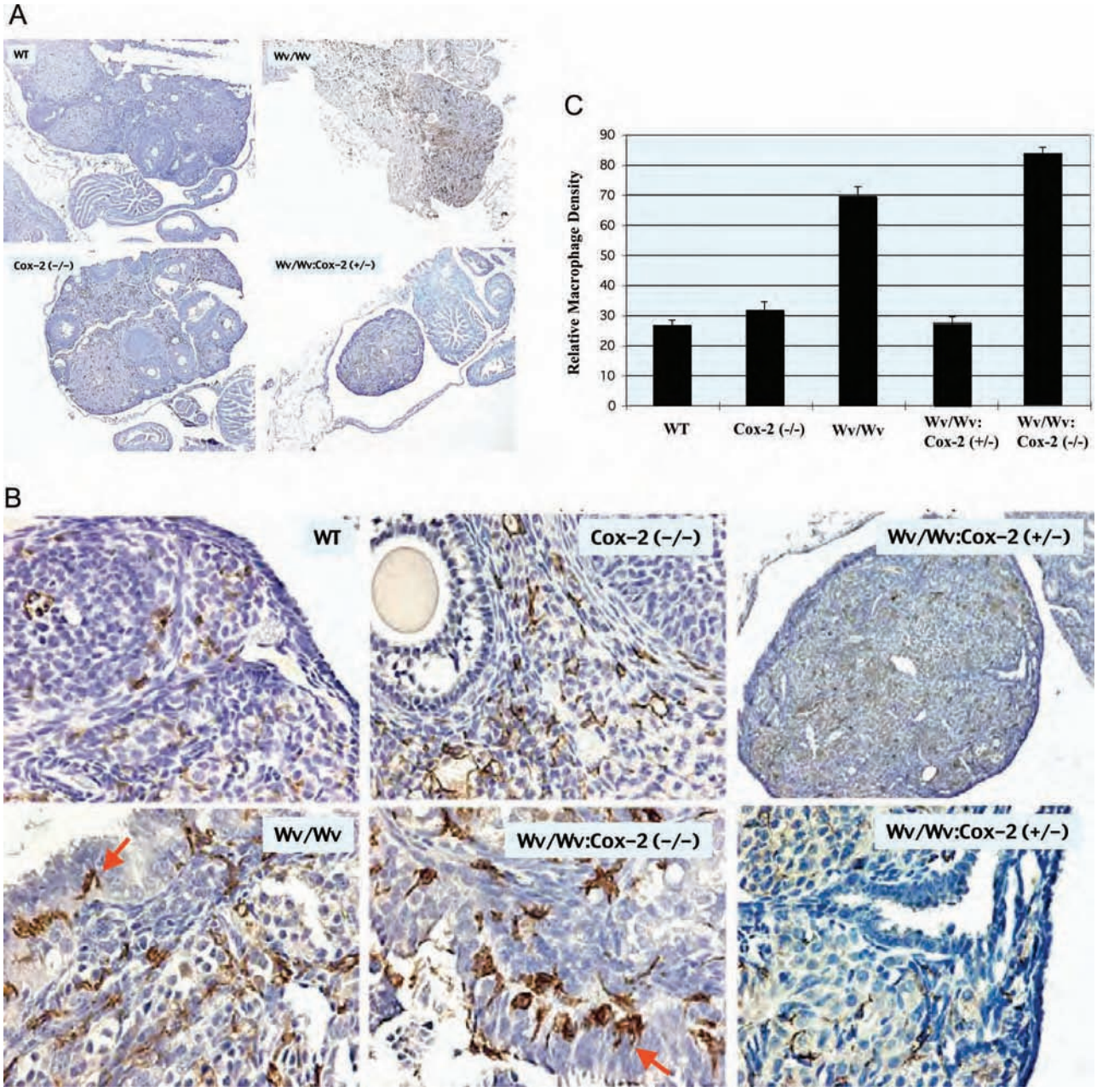

Figure 5. Ovarian macrophage density in Wv and Cox-2 mutant mice. **A:** Macrophages were identified by staining with F4/80. **B:** Examples of a higher magnification of ovaries stained with the F4/80 macrophage marker are shown. The **arrow** indicates macrophages infiltrating epithelia. **C:** Macrophage density was quantitated by counting five fields each of three ovaries from each genotype. Averages of five countings with SDs are shown. Student's *t*-test indicates that macrophage numbers are not statistically significant between WT, Cox-2 (−/−), and Wv/Wv:Cox-2 (+/−) ($P > 0.05$), but when the values of these three genotypes compared with those of Wv/Wv or Wv/Wv:Cox-2 (−/−), the difference is statistically significant ($P < 0.001$). Original magnifications: ×40 (**A**); ×200 (**B**).

Pharmacological Inhibitors of Coxs Effectively Prevent Ovarian Epithelial Transformation and Tumorigenesis

We then tested whether the effect of reducing the Cox-2 gene dosage on ovarian tumor phenotype can be achieved by using pharmacological agents. Based on previous studies and our own toxicity study specifically in Wv/Wv female mice, nontoxic dosages of celecoxib, a Cox-2-specific inhibitor, and indomethacin, a nonselective COX inhibitor, were determined to be 200 and 30 μg/day, respectively. When the Wv/Wv female mice received these inhibitors starting at 4 weeks of age, a significant reduction in tumor phenotype was observed at the age of 3 months in all ovaries, and some ovaries were devoid of tubular adenomas (Figure 6). In three of eight Wv/Wv mice, celecoxib feeding prevented the ovarian tumor phenotype; and in the indomethacin-treated group, five of seven mice were rescued. All ovaries of the five control Wv/Wv littermates that were maintained identically

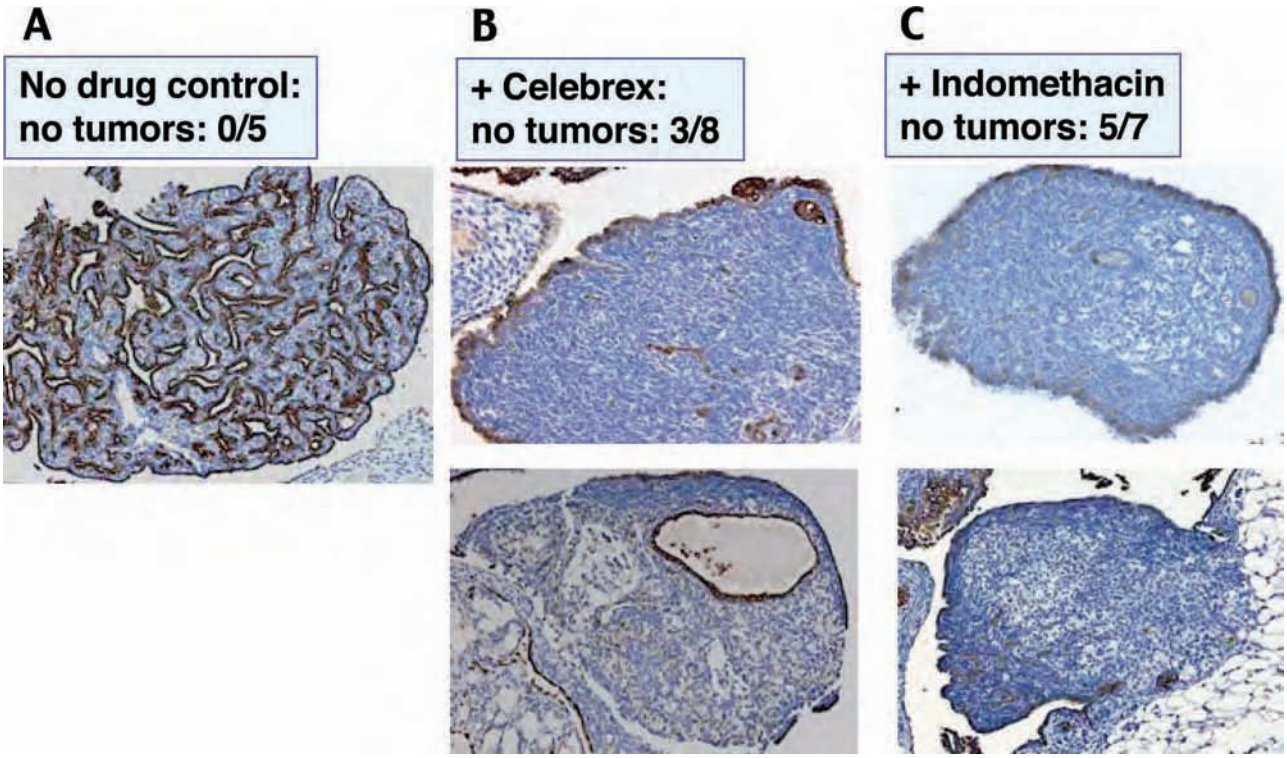

Figure 6. Rescue of Wv/Wv mouse ovarian tubular adenoma phenotype by celecoxib (Celebrex) and indomethacin administration. The representative ovarian morphology from Wv/Wv mice controls (**A**) and those fed with celecoxib (**B**) or indomethacin (**C**) was compared. Cytokeratin-8 (Troma-1) staining was used to identify epithelial cells. The *P* values generated from the χ^2 test (or Fisher's exact test) are 0.2308, 0.0278, and 0.3147, respectively, for the comparisons between celecoxib treatment versus control, indomethacin treatment versus control, and celecoxib versus indomethacin treatment. Thus in this experiment only the difference between indomethacin treatment versus control reaches statistical significance. One example of wild type and two examples of treatment with celecoxib and indomethacin are shown. Original magnifications, ×40.

but did not receive inhibitors showed ovarian tubular adenomas. Only the comparison between controls and indomethacin-treated mice in this experiment reaches statistical significance. However, considering that ovarian tumors were found in all of the more than 300 Wv female mice analyzed previously, the observed reduction in tumor development by both celecoxib and indomethacin is likely significant. Thus in this preliminary study including only a small number of animals, we found that Cox inhibitors are able to prevent ovarian epithelial morphological transformation and tumor phenotypes. Inhibition of both Cox-1 and Cox-2 with indomethacin seems more effective than inhibition of Cox-2 alone with celecoxib, but the difference did not reach statistical significance. The number of animals used in these experiments is relatively small, and additional confirmation is needed. When indomethacin was given for a period of 1 month to Wv/Wv mice at 3 months of age when ovarian tumors were already established, the tumors were not reduced compared with controls (not shown), suggesting inhibition of Coxs prevents the development of ovarian tumors but has no suppressive effect on established tumors.

Discussion

In the present study, we demonstrate that a reduction of Cox-2 gene dosage rescued the ovarian aging phenotype of the Wv/Wv mice, whereas homozygous deletion of Cox-2 gene was less effective in reducing the formation of these epithelial lesions in the ovaries because Cox-2 elimination was accompanied by an increase in ovarian Cox-1 expression and PGE_2 synthesis. The tumor phenotype was also reduced by the use of pharmacological agents to inhibit Cox-1 and/or Cox-2. These findings suggest that increased Cox-1/Cox-2 activity contributes to the preneoplastic morphological changes of the ovarian surface epithelium, and the tumor phenotype can be reversed by a reduction of Cox gene dosage either by genetic or pharmacological approaches. The results suggest the inflammatory environment of the germ cell-depleted ovaries stimulates epithelial morphological changes and tumor development, and this circumstance provides an excellent example of how inflammation promotes cancer. This study provides a model to study the links between reproductive factors, inflammation, and ovarian tumor development.

Inflammatory Environment in Postmenopausal Ovaries

The link between inflammation and cancer is well recognized: chronic inflammation induced by infections or other chemical and pathological factors creates an inflammatory microenvironment in the tissue, which is composed of epithelial cells, stromal cells, leukocytes, and macrophages. These cells are networked by autocrine

and paracrine interactions mediated by proinflammatory cytokines such as tumor necrosis factor-α and interleukin-β.[34,35] Signal pathways involving nuclear factor-κB, tumor necrosis factor-α, and Coxs are known to be involved in both inflammation and cancer. The inflammatory mediators such as proteolytic enzymes, prostaglandins, and diverse reactive oxygen and nitrogen species create a tumor-promoting environment in which transformed (altered by either genetic or epigenetic means) epithelial cells thrive and are selected to expand.[34,35] Coxs, Cox-1 and/or Cox-2, play crucial roles in regulation of inflammation and are thought to be targets of the anti-inflammatory activity of nonsteroidal anti-inflammatory drugs for cancer prevention.[18,19,23]

Gene knockout mice suggest that both Cox-1 and Cox-2 are dispensable for development and have verified the role of Cox-1 and Cox-2 in inflammation that counters host infection by microorganisms.[29,30] Another established physiological function for Cox activity is in reproduction, especially ovulation,[14–16] which is considered an inflammatory-like process.[17] Ovarian Cox-2 is induced by the periodical surge of gonadotropins and plays an important and necessary role in the proteolysis and tissue remodeling that precedes the ovulatory rupture of the follicle and ovarian surface to release the ova.[36] The elevated gonadotropin level in postmenopause, although it does not cause ovarian surface rupture as in ovulation, still stimulates the ovulation-like inflammatory process and Cox expression.[28] The Wv mouse model simulates the postmenopausal ovarian inflammatory conditions as indicated by the increased expression of ovarian Cox-1 and Cox-2 and macrophage infiltration.

We propose that this ovarian inflammatory environment promotes tissue remodeling and perturbation or disruption of epithelial structure, leading to epithelial morphological transformation and the development of the ovarian tubular adenomas in the Wv mice. This mechanism may explain gonadotropin stimulation as an etiological factor for an increased ovarian cancer risk. The ovarian tumor in Wv mouse also represents a unique model in which a physiological inflammatory environment promotes tumor development.

Germ Cell-Deficient Wv Mice as Models for Reproductive Factors in Ovarian Cancer Etiology

Although the ovarian tumors may invade adjacent tissues and fat pads at later stages,[9] we have not observed any shedding of tumor cells and the formation of ascites in Wv mice. Although the ovarian tubular adenomas are derived from ovarian surface epithelial cells, the histology of the tumors differs from human epithelial ovarian cancer. Thus, the Wv mouse is not a model for human malignant epithelial ovarian cancer.

Nevertheless, the ovarian epithelial morphological transformation in Wv mice resembles ovarian morphological changes associated with reproductive aging and perimenopausal gonadotropin stimulation. Most likely, the Wv mouse model mimics certain biological aspects of ovarian cancer risk associated with menopause and gonadotropin stimulation. The ovarian tumors in Wv mice are associated with excessive hormonal stimulation but lack genetic mutations that are commonly found in human ovarian cancer, such as Ras and B-Raf mutations in borderline tumors, p53 mutations in malignant ovarian cancer, and pten mutations in the endometrial subtype of ovarian carcinomas.[37] In the last few years, a number of technical breakthroughs have led to the establishment of several mouse models for ovarian cancer. First, a genetically defined model of ovarian cancer was established by Orsulic and colleagues,[38] in which mouse ovarian surface epithelial cells were transfected with defined genetic changes such as k-Ras, v-Akt, v-myc, and so forth. The cells were then reimplanted into the ovarian bursa and malignant ovarian tumors developed. Using the MIS II R promoter, a mainly ovarian-restricted transcript, Connolly and colleagues[39] developed the T-antigen transgenic line that develops malignant bilateral ovarian tumors. Presumably, T-antigen expression results in the inactivation of both p53 and Rb. Indeed, using adenoviral delivery of cre to ovaries of mice with floxed p53 and Rb, Flesken-Nikitin and colleagues[40] demonstrated the development of malignant ovarian tumors when both p53 and Rb are deleted. Most recently, mice with conditional expression of K-ras and deletion of pten in ovarian surface epithelial cells were found to develop endometriosis and endometrioid carcinomas.[41] Because both mutations are present in endometriosis and endometrioid ovarian cancer in humans, this model seems to recapitulate the genotype and histomorphology associated with the human disease. There are likely additional ovarian cancer animal models that are not mentioned here. These genetic models have provided persuasive evidence for the relevance of these mutations in ovarian carcinogenesis but, nevertheless, have not yet incorporated components related to the etiology of ovarian cancer.

To reconcile the ovarian etiology related to gonadotropin stimulation and postmenopausal biology with genetic mutations in the development of ovarian cancer, it may be proposed that the postmenopausal gonadotropin stimulated epithelial proliferation and morphological transformation may select and promote the expansion of cells with genetic mutations. Mice with a p53 mutation alone do not develop ovarian epithelial tumors, even when ovaries with mutant p53 are transplanted into wild-type hosts to bypass the development of sarcomas and lymphomas in the p53 mutant background.[42] As a prediction, if additional genetic mutations (such as a p53 mutation) are added to the Wv/Wv females, malignant ovarian carcinomas may develop. Preliminary results of these experiments in our laboratory are very suggestive, but a thorough analysis of these mice with compound genetic mutations will take its course.

Implication of the Cox-2 Gene Dosage Effect

Unexpectedly, a reduction of Cox-2 gene dosage by heterozygous deletion is more effective than homozy-

gous deletion in preventing the ovarian epithelial morphological change and the formation of tubular adenomas in Wv/Wv mice. This seems attributable to a compensatory increase in ovarian Cox-1 when Cox-2 is completely eliminated. Although a compensation between the expression of Cox-1 and Cox-2 was not observed in Cox-2 gene knockout mice initially,[14,29] it has since been noted,[43] and Cox-1 can substitute for Cox-2 in ovulation in certain genetic backgrounds.[44] Thus, the roles of both Cox-1 and Cox-2 in promoting ovarian tumor development suggest drugs that inhibit both Coxs may be more effective than specific inhibitors of Cox-2 in reducing the development of ovarian tumors.

Moreover, a reduction of the Cox-2 gene copy number is sufficient to reduce the ovarian tumor phenotype in the Wv mice is unique, because reduction of one gene copy number seldom shows a phenotype in gene knockout mice. This dosage-dependent effect of Cox-2 in promoting ovarian tumor development may have crucial implications in strategies using inhibitors as preventive agents for ovarian cancer. It suggests that the use of a low dosage of the drugs to reduce Cox-2 (and Cox-1) activity, rather than a complete suppression of the activity, may be sufficient to reduce tumor incidence.

Interestingly, Cox-1, instead of Cox-2, was found to be overexpressed in human ovarian cancer cancer.[31,32] The increased Cox-1 expression was observed in several mouse ovarian tumor models models,[33] and we also found that Cox-1 is increased in Wv mouse ovarian tumors. Thus, Cox-2 may be important in tumor initiation, and Cox-1 increase may be important for progression and malignancy.

In summary, this study indicates that inhibition of Coxs reduces the morphological perturbation of ovarian surface epithelium induced by increased gonadotropins in Wv mice, which model postmenopausal ovarian biology, and provides further rationale and support for the use of nonsteroidal anti-inflammatory drugs and specific Cox-1 and/or Cox-2 inhibitors for ovarian cancer prevention in peri- and postmenopausal women. In parallel, we found that in human ovaries, peri- and postmenopausal age is the key determinant of ovarian surface epithelial preneoplastic morphological changes in populations with and without BRCA mutations.[2] Thus, the circumstances in Wv mice are highly pertinent to menopausal women in both biology and pathology. The findings that a reduction instead of complete inhibition of Cox-2 is effective in the suppression of ovarian epithelial lesions and that a compensatory mechanism between the expression of Cox-1 and Cox-2 may offer new strategies for clinical intervention.

Acknowledgments

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References

1. Gosden RG: Biology of Menopause: The Causes and Consequences of Ovarian Aging. Edited by RG Gosden. New York, Academic Press, 1985
2. Cai KQ, Klein-Szanto A, Karthik D, Edelson M, Daly MB, Ozols RF, Lynch HT, Godwin AK, Xu XX: Age-dependent morphological alterations of human ovaries from populations with and without BRCA mutations. Gynecol Oncol 2006, 103:719–728
3. Nicosia SV: The aging ovary. Med Clin North Am 1987, 71:1–9
4. Salazar H, Godwin AK, Daly MB, Laub PB, Hogan WM, Rosenblum N, Boente MP, Lynch HT, Hamilton TC: Microscopic benign and invasive malignant neoplasms and a cancer-prone phenotype in prophylactic oophorectomies. J Natl Cancer Inst 1996, 88:1810–1820
5. Mintz B: Embryological development of primordial germ-cells in the mouse: influence of a new mutation, Wj. J Embryol Exp Morphol 1957, 5:396–406
6. Dubreuil P, Rottapel R, Reith AD, Forrester L, Bernstein A: The mouse W/c-kit locus. A mammalian gene that controls the development of three distinct cell lineages. Ann NY Acad Sci 1990, 599:58–65
7. Nocka K, Tan JC, Chiu E, Chu TY, Ray P, Traktman P, Besmer P: Molecular bases of dominant negative and loss of function mutations at the murine c-kit/white spotting locus: w37, Wv, W41 and W. EMBO J 1990, 9:1805–1813
8. Reith AD, Rottapel R, Giddens E, Brady C, Forrester L, Bernstein A: W mutant mice with mild or severe developmental defects contain distinct point mutations in the kinase domain of the c-kit receptor. Genes Dev 1990, 4:390–400
9. Murphy ED: Hyperplastic and early neoplastic changes in the ovaries of mice after genic deletion of germ cells. J Natl Cancer Inst 1972, 48:1283–1295
10. Eldridge JC, McPherson III JC, Mahesh VB: Maturation of the negative feedback control of gonadotropin secretion in the female rat. Endocrinology 1974, 94:1536–1540
11. Murphy ED, Beamer WG: Plasma gonadotropin levels during early stages of ovarian tumorigenesis in mice of the W x/W u genotype. Cancer Res 1973, 33:721–723
12. Blaakaer J, Baeksted M, Micic S, Albrectsen P, Rygaard J, Bock J: Gonadotropin-releasing hormone agonist suppression of ovarian tumorigenesis in mice of the Wx/Wv genotype. Biol Reprod 1995, 53:775–779
13. Cramer DW, Welch WR: Determinants of ovarian cancer risk. II. Inferences regarding pathogenesis. J Natl Cancer Inst 1983, 71:717–721
14. Lim H, Paria BC, Das SK, Dinchuk JE, Langenbach R, Trzaskos JM, Dey SK: Multiple female reproductive failures in cyclooxygenase 2-deficient mice. Cell 1997, 91:197–208
15. Davis BJ, Lennard DE, Lee CA, Tiano HF, Morham SG, Wetsel WC, Langenbach R: Anovulation in cyclooxygenase-2-deficient mice is restored by prostaglandin E2 and interleukin-1beta. Endocrinology 1999, 140:2685–2695
16. Reese J, Zhao X, Ma WG, Brown N, Maziasz TJ, Dey SK: Comparative analysis of pharmacologic and/or genetic disruption of cyclooxygenase-1 and cyclooxygenase-2 function in female reproduction in mice. Endocrinology 2001, 142:3198–3206
17. Richards JS, Russell DL, Ochsner S, Espey LL: Ovulation: new dimensions and new regulators of the inflammatory-like response. Annu Rev Physiol 2002, 64:69–92
18. Williams CS, Mann M, DuBois RN: The role of cyclooxygenases

in inflammation, cancer, and development. Oncogene 1999, 18:7908–7916
19. Prescott SM, Fitzpatrick FA: Cyclooxygenase-2 and carcinogenesis. Biochim Biophys Acta 2000, 1470:M69–M78
20. Oshima M, Dinchuk JE, Kargman SL, Oshima H, Hancock B, Kwong E, Trzaskos JM, Evans JF, Taketo MM: Suppression of intestinal polyposis in Apc delta716 knockout mice by inhibition of cyclooxygenase 2 (COX-2). Cell 1996, 87:803–809
21. Lal G, Ash C, Hay K, Redston M, Kwong E, Hancock B, Mak T, Kargman S, Evans JF, Gallinger S: Suppression of intestinal polyps in Msh2-deficient and non-Msh2-deficient multiple intestinal neoplasia mice by a specific cyclooxygenase-2 inhibitor and by a dual cyclooxygenase-1/2 inhibitor. Cancer Res 2001, 61:6131–6136
22. Chulada PC, Thompson MB, Mahler JF, Doyle CM, Gaul BW, Lee C, Tiano HF, Morham SG, Smithies O, Langenbach R: Genetic disruption of Ptgs-1, as well as Ptgs-2, reduces intestinal tumorigenesis in Min mice. Cancer Res 2000, 60:4705–4708
23. Subbaramaiah K, Dannenberg A: Cyclooxygenase 2: a molecular target for cancer prevention and treatment. Trends Pharmacol Sci 2003, 24:96–102
24. Steinbach G, Lynch PM, Phillips RK, Wallace MH, Hawk E, Gordon GB, Wakabayashi N, Saunders B, Shen Y, Fujimura T, Su LK, Levin B: The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. N Engl J Med 2000, 342:1946–1952
25. Cramer D, Harlow B, Titus-Ernstaff L, Bohlke K, Welch WR, Greenberg ER: Over-the-counter analgesics and risk of ovarian cancer. Lancet 1998, 351:104–107
26. Sørensen HT, Friis S, Norgard B, Mellemkjaer L, Blot WJ, McLaughlin JK, Ekbom A, Baron JA: Risk of cancer in a large cohort of nonaspirin NSAID users: a population-based study. Br J Cancer 2003, 88:1687–1692
27. Schildkraut JM, Moorman PG, Halabi S, Calingaert B, Marks JR, Berchuck A: Analgesic drug use and risk of ovarian cancer. Epidemiology 2006, 17:104–107
28. Smith ER, Daly MB, Xu XX: A mechanism for Cox-2 inhibitor anti-inflammatory activity in chemoprevention of epithelial cancers. Cancer Epidemiol Biomarkers Prev 2004, 13:144–145
29. Morham SG, Langenbach R, Loftin CD, Tiano HF, Vouloumanos N, Jennette JC, Mahler JF, Kluckman KD, Ledford A, Lee CA, Smithies O: Prostaglandin synthase 2 gene disruption causes severe renal pathology in the mouse. Cell 1995, 83:473–482
30. Smith WL, Langenbach R: Why there are two cyclooxygenase isozymes. J Clin Invest 2001, 107:1491–1495
31. Gupta RA, Tejada LV, Tong BJ, Das SK, Morrow JD, Dey SK, DuBois RN: Cyclooxygenase-1 is overexpressed and promotes angiogenic growth factor production in ovarian cancer. Cancer Res 2003, 63:906–911
32. Daikoku T, Wang D, Tranguch S, Morrow JD, Orsulic S, DuBois RN, Dey SK: Cyclooxygenase-1 is a potential target for prevention and treatment of ovarian epithelial cancer. Cancer Res 2005, 65:3735–3744
33. Daikoku T, Tranguch S, Trofimova IN, Dinulescu DM, Jacks T, Nikitin AY, Connolly DC, Dey SK: Cyclooxygenase-1 is overexpressed in multiple genetically engineered mouse models of epithelial ovarian cancer. Cancer Res 2006, 66:2527–2531
34. Balkwill F, Coussens LM: Cancer: an inflammatory link. Nature 2004, 431:405–406
35. Clevers H: At the crossroads of inflammation and cancer. Cell 2004, 118:671–674
36. Tsafriri A, Reich R: Molecular aspects of mammalian ovulation. Exp Clin Endocrinol Diabetes 1999, 107:1–11
37. Ozols RF, Bookman MA, Connolly DC, Daly MB, Godwin AK, Schilder RJ, Xu XX, Hamilton TC: Focus on epithelial ovarian cancer. Cancer Cell 2004, 5:19–24
38. Orsulic S, Li Y, Soslow RA, Vitale-Cross LA, Gutkind JS, Varmus HE: Induction of ovarian cancer by defined multiple genetic changes in a mouse model system. Cancer Cell 2002, 1:53–62
39. Connolly DC, Bao R, Nikitin AY, Stephens KC, Poole TW, Hua X, Harris SS, Vanderhyden BC, Hamilton TC: Female mice chimeric for expression of the simian virus 40 TAg under control of the MISIIR promoter develop epithelial ovarian cancer. Cancer Res 2003, 63:1389–1397
40. Flesken-Nikitin A, Choi KC, Eng JP, Shmidt EN, Nikitin AY: Induction of carcinogenesis by concurrent inactivation of p53 and Rb1 in the mouse ovarian surface epithelium. Cancer Res 2003, 63:3459–3463
41. Dinulescu DM, Ince TA, Quade BJ, Shafer SA, Crowley D, Jacks T: Role of K-ras and Pten in the development of mouse models of endometriosis and endometrioid ovarian cancer. Nat Med 2005, 11:63–70
42. Chen CM, Chang JL, Behringer RR: Tumor formation in p53 mutant ovaries transplanted into wild-type female hosts. Oncogene 2004, 23:7722–7725
43. Kirtikara K, Morham SG, Raghow R, Laulederkind SJ, Kanekura T, Goorha S, Ballou LR: Compensatory prostaglandin E2 biosynthesis in cyclooxygenase 1 or 2 null cells. J Exp Med 1998, 187:517–523
44. Wang H, Ma WG, Tejada L, Zhang H, Morrow JD, Das SK, Dey SK: Rescue of female infertility from the loss of cyclooxygenase-2 by compensatory up-regulation of cyclooxygenase-1 is a function of genetic makeup. J Biol Chem 2004, 279:10649–10658

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Prominent Expression of Metalloproteinases in Early Stages of Ovarian Tumorigenesis

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The role for matrix metalloproteinases (MMPs) in tumor cells invasion and metastasis is well established, and expression of MMPs is recognized as an indication of tumor cell malignancy. Previous studies suggest that the degradation of the basement membrane is a crucial early step in epithelial transformation and ovarian tumorigenesis. Thus, MMPs may also express and exert a role in preneoplastic lesions of ovarian tissues. We investigated the expression of the major metalloproteinases, gelatinase A, 72 kDa type IV collagenase (MMP-2), and gelatinase B, 92 kDa type IV collagenase (MMP-9), and the presence of basement membrane in ovarian tumors and tissues from prophylactic oophorectomies using immunostaining. MMP expression was also characterized in a panel of ovarian cancer cell lines and several nontumorigenic ovarian surface epithelial primary cells by zymography, Northern, and Western blots. We found, surprisingly, that MMP-2 and MMP-9 are expressed more frequently in early lesions than in established carcinomas. No correlation was found between the expression of MMPs and tumor grades or stages. In preneoplastic lesions, MMP-2 or MMP-9 expression often associates with the absence of basement membrane and morphological alterations. MMP-2 is often expressed in nontumorigenic ovarian surface epithelial cells but reduced or absent in cancer cells. Thus, we conclude that MMPs expression does not correlate with the malignancy of ovarian epithelial cells as generally thought. Rather, increased metalloproteinase expression is an early event in ovarian tumorigenesis and associates with the loss of epithelial basement membrane and morphological transformation. We propose that the increased MMP activity is an etiological factor for ovarian cancer risk. We found that MMPs expression does not correlate with the malignancy of ovarian epithelial cells as generally thought. Rather, increased metalloproteinase expression is an early event in ovarian tumorigenesis. The finding suggests roles of MMP in tumor initiation in addition to invasion, and may impact on the strategy for use of MMP inhibitors in cancer prevention.

Key words: ovarian cancer; preneoplastic changes; MMPs; epithelium; basement membrane

INTRODUCTION

The ovarian surface epithelium is a monolayer consisting of flat to cuboidal cells, which are organized by a layer of basement membrane composed primarily of collagen IV and laminin [1]. The basement membrane plays an important role in epithelial cell organization, differentiation, proliferation, and transformation [2,3]. Previous studies in our lab suggest that loss of the epithelial basement membrane is a crucial early step in morphological transformation and tumor initiation of the ovarian surface epithelia [4,5].

The integrity of the basement membrane is regulated both by proteolytic degradation and synthesis/remodeling, and is a critical regulatory factor for epithelial cells during many physiological and pathological conditions. The basement membrane is degraded mainly by a family of matrix metalloproteinases (MMPs) [6,7]: zinc-dependent endopeptidases. In ovarian epithelial cells, gelatinase A, 72 kDa type IV collagenase (MMP-2) and gelatinase B, 92 kDa type IV collagenase (MMP-9) are the two major MMPs activities detected by zymography [8,9].

In human ovarian surface epithelial cells, MMP-9 expression was found to increase dramatically following stimulation by either tumor necrosis factor alpha (TNF-α) or interleukin-1 beta (IL-1β) [9]. MMP-2 is constitutively produced by human ovarian surface epithelial (HOSE) cells but not regulated by TNF-α, IL-1β, or other factors and hormones that

Abbreviations: Dab2, Disabled-2; HIO cells, human ''immortalized'' ovarian surface epithelial cells; IL-1β, interleukin-1 beta; LMP, low-malignant potential; MMPs, matrix metalloproteinases; MMP-2, gelatinase A, 72 kDa type IV collagenase; MMP-9, gelatinase B, 92 kDa type IV collagenase; TMAs, tissue microarrays; TNF-α, tumor necrosis factor alpha.

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potentially involved in ovulation [10]. Regulation of MMP-9 by TNF-α or IL-1β is likely critical for proteolytic activation that occurs during tissue rupture in ovulation. It is also speculated that loss of basement membrane in ovarian surface epithelial transformation may have a similar mechanism as the loss of surface epithelial basement membrane in ovulation [5].

Morphological changes of ovarian surface epithelia such as surface papillomatosis, inclusion cysts, surface epithelial invaginations, and nuclear pleomorphism or stratification of surface epithelial cells have been postulated by various investigators to be potential premalignant histologic features [10–12]. Such morphological alterations can often be observed in ovaries prophylactically removed from women with an inherited predisposition for ovarian cancer [12,13]. Morphologically benign ovarian surface epithelia found adjacent to neoplastic cells are also likely preneoplastic lesions [14–16]. These putative preneoplastic lesions may provide materials to study the molecular mechanisms and alterations of gene expression involved in epithelial morphological transformation and the development of ovarian cancer.

Among the markers and genes that associate with ovarian cancer, a consistent finding is that the transformation of monolayer ovarian epithelia to stratified or neoplastic epithelial lesions closely correlates with the loss of Disabled-2 (Dab2) [14,15], a putative tumor suppressor of ovarian cancer [15,17,18]. A role for Dab2 in epithelial structure organization, or so-called epithelial positioning has been proposed [17], and the disruption of visceral endoderm, an epithelium in early embryos, in Dab2 knockout mice [19] supports the idea that Dab2 has a role in epithelial organization and the loss of Dab2 may be a causative factor in epithelial morphological transformation in ovarian cancer. Additional observation suggests that loss of Dab2 expression is not sufficient, and loss of basement membrane also contributes to ovarian surface epithelial morphological transformation [5,14].

In the current study, we investigated in ovarian tumor tissues, prophylactic oophorectomies, and both nontumorigenic and cancerous ovarian surface epithelial cells to determine if MMP-2 and MMP-9 expression contributes to the observed basement membrane loss in the early step of ovarian tumorigenesis. We also investigated the mechanism for the altered expression regulation of MMP-2 and MMP-9.

MATERIALS AND METHODS

Ovarian Tissues and Tumor Specimens and Immunohistochemistry

Cancerous and benign ovarian specimens were obtained from patients who underwent surgical resection at Fox Chase Cancer Center (Table 1). The 3 ovarian tumor tissue microarrays (TMAs) and 20 prophylactic oophorectomies were provided by the Tumor Bank Facility of Fox Chase Cancer Center. We also used a collection of 38 separate cases of archived ovarian tumor tissue blocks. The tumors were histologically classified according to the World Heath Organization (WHO) classification and the surgical stages were determined according to the

Table 1. List of Tissues Used for Analysis

Tissue	Number	Details
Control ovaries	10	From noncancer disease (benign uterine lesions)
Prophylactic oophorectomies	20	From high-risk population
TMA	68	(170 Cores, 68 informative from 86 total cases)
		47 serous adenocarcinomas
		3 endometrioid adenocarcinomas
		1 mucinous adenocarcinomas
		3 clear-cell carcinomas
		1 malignant mixed mesodermal tumor
		3 metastatic ovarian carcinomas
		5 undifferentiated carcinomas
		2 serous LMP tumors
		2 mucinous LMP tumors
		1 benign serous cystadenoma
Ovarian tumor blocks	38	
		18 serous adenocarcinomas
		5 mucinous adenocarcinomas
		2 endometrioid adenocarcinomas
		3 clear cell carcinomas
		6 serous LMP
		3 mucinous LMP
		1 benign fibrous cystadenoma

classification of International Federation of Gynecology and Obstetrics (FIGO).

Immunostaining was performed with the mouse DAKO Envision TM+ System and the Peroxidase (DAB) Kit (Dako Corporation, Carpinteria, CA) as previously described [5]. The antibodies used include: anti-MMP-9 (1:1000 dilution, clone $15W_2$, Novacastra Laboratories, New Castle, UK), anti-MMP-2 (Ab-4. 1:300 dilution, clone A-Gel VC2, NeoMarkers, Fremont, CA), anti-collagen IV (1:100 dilution, clone CIV22, Dako), and anti-Dab2/p96 (1:400 dilution, clone 52, BD Transduction Laboratories, Lexington, KY). Negative controls were prepared by replacing the primary antibody with mouse nonimmunized IgG. Evaluation of the staining intensity and characteristics was performed independently by two pathologists in a blinded manner. Tumors with more than 10% of tumor cells or stromal area showing strong to moderate staining are scored as positive.

For PAS staining, the sections were first deparaffinized and hydrated with water, and then were oxidized in 0.5% periodic acid solution for 5 min and kept in Schiff reagent (Sigma, St. Louis, MO) for 15 min. The sections were then counterstained with Mayer's hematoxylin for 1 min and then dehydrated and sealed with coverslips.

Cell Culture, Western, and Northern Blots

The panel of human ''immortalized'' ovarian (HIO) epithelial cell lines [9,20], two preparations of primary human ovarian surface epithelial cells [9,20], and ovarian cancer cell lines were analyzed. Ovarian cancer cells OVCAR2, -3, -4, -5, -8, and -10 were collected in Dr. Andrew Godwin's lab and were used previously [9,20]. Other ovarian cancer cells including A1847, A2780, ES2, OV1016, PEO-1, and SKOV3 were purchased from American Type Culture Collection (ATCC), where information about these ovarian cancer cells is available. The primary and HIO-ovarian surface epithelial cells were cultured in medium 199 and MCDB 105 (1:1) supplemented with 15% FBS, 0.25 U/mL of insulin, 2 mM L-glutamine, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. Primary cells cultured between passages 2 and 4 were used for experiments. The ovarian cancer cell lines were cultured in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL of penicillin, and 100 μg/mL of streptomycin. The cultures were maintained in a humidified atmosphere of 95% air/5% CO_2 at 37°C.

Confluent cells cultured in 60-mm plates were washed twice with PBS and switched to the same culture medium without serum for 18 h to obtain conditioned medium, which was concentrated fourfold with Centricon 10 concentrators (Amicon, Beverly, MA), and then the concentrated conditioned medium was mixed with 4× sample buffer at 3:1 ratio. Samples of total cell lysates (about 100 μg) and concentrated conditioned medium were analyzed by Western blot with antibodies for MMP-9 or MMP-2 (Oncogene, Inc., San Diego, CA). Northern blot analysis was performed as previously described [20]. Probes were EST clones obtained from ATCC and were verified by sequencing.

Suppression of Gene Expression by siRNA Approach

Reduced/suppressed of GATA6 expression in ovarian cancer cells was performed according to previously published procedure [20]. Briefly, the pSuppressorNeo vector (Imgenex, San Diego, CA) with or without siRNA insert targeting GATA6 were transfected into GATA6-positive ES2 or HIO-118 cells with Mirus transIT-LT1 reagent (Mirus corporation, Madison, WI). Following a 24-h incubation, fresh medium was added. The condition medium was used to determine MMP activity by zymography, the cells were used for Western blot analysis to evaluate gene suppression.

With green fluorescence protein (GFP) expressing vector for co-transfection, the transfection efficiency was determined to be around 70% after 24 h, which was determined to be the optimal time in suppression of GATA6 expression by transient transfection.

Gelatin Zymography

SDS-polyacrylamide (10%) gels copolymerized with 1 mg/mL gelatin were used to detect both latent and activated forms of gelatinases. An aliquot of about 50 μg of total protein from concentrated conditioned medium was separated by electrophoresis under nonreducing conditions. The gels were washed twice with 2.5% Triton X-100 for 30 min at room temperature with constant shaking to remove SDS. Gels were subsequently incubated overnight at 37°C in the development buffer [50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 5 mM $CaCl_2$, 1% (v/v) Triton X-100, 0.02% Brij-35]. The enzymatic activity was visualized by staining the gels with 0.1% (w/v) Coomassie blue G-250 in 40% (v/v) methanol/10% (v/v) glacial acetic acid at room temperature for 60 min following by destaining in 10% (v/v) methanol with 7% (v/v) acetic acid.

RESULTS

Expression of MMP-2 and MMP-9 in Ovarian Tumors

To examine the idea that expression of MMPs may be the cause in the loss of basement membrane and morphological transformation, we investigated the expression of MMP-9 and MMP-2 in a collection of ovarian tissues (Table 1) and cells lines. Typical normal ovarian surface epithelia, representative of 10 control ovaries, do not express MMP-2 or MMP-9, and the surface epithelial basement membrane is well-defined as revealed by staining with Periodic

Acid Schiff (PAS) reagent or collagen IV (Figure 1A) (arrows). PAS stains glycoproteins in the basement membrane.

Tissue microarrays (TMAs) of ovarian tumors were stained with MMP-2 and MMP-9 antibodies. After excluding defective cores and combining duplications of cores, we obtained results from a total of 68 informative cases. The staining patterns of MMP-2 and MMP-9 are different and quite unique. Of the 22 out of 68 tumors (32.3%) classified as MMP-9 positive, MMP-9 staining is detected in stroma surrounding the tumor epithelial cells and is not found in the neoplastic epithelial cells (Figure 1B). In contrast, MMP-2 staining is present in both epithelial and/or stromal compartments in 30 out of the 68 tumors (44.1%). Of these 30 MMP-2 positive tumors, heterogeneous MMP-2 staining is detected only in neoplastic epithelial cells in 15 cases, only in stromal compartments in 11 cases, and in both tumor and stroma cells in 4 (13.3%) cases. Strong MMP-2 staining was also observed in endothelial cells and smooth muscle cells of the blood vessels. Examples of the immunostaining are shown in Figure 1B.

The unique staining patterns of MMP-2 and MMP-9 were also found in the whole sections of the 38 ovarian tumor tissues. In several ovarian carcinomas, it was observed that MMP-2 is strongly expressed around the edges of tumor nodules bordering stroma (Figure 1C). Examples of MMP-2, MMP-9, and collagen IV immunostaining for ovarian carcinomas of serous and other histological subtypes (Figure 1C) are shown.

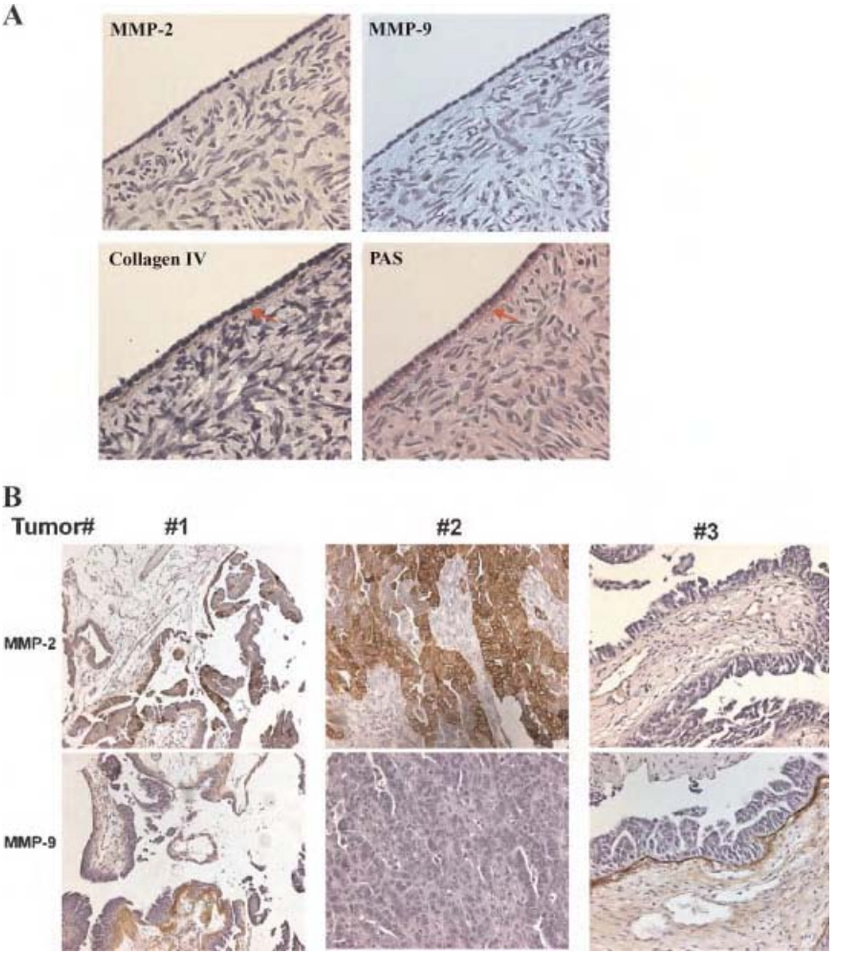

Figure 1. Examples of immunostaining of ovarian tissues. (A) Characteristics of a normal ovarian surface epithelium: immunostaining for MMP-2, MMP-9, and collagen IV. PAS and coolagen IV positive staining show the presence of an intact basement membrane as indicated by arrows (original magnification: 400×). (B) Representative examples for immunostaining of MMP-2 and MMP-9. Examples of MMP-2 and MMP-9 staining patterns of three tumors in ovarian tissue microarrays are shown. Tumor 1: A serous carcinoma with positive cytoplasmic staining of MMP-2 in tumor cells and positive staining of MMP-9 in the stroma surrounding the cancer cells. Tumor 2: A serous carcinoma with positive cytoplasmic staining of MMP-2 in tumor cells and staining of MMP-9 is negative. Tumor 3: An ovarian tumor with negative staining of MMP-2 and positive staining of MMP-9 in the stroma surrounding the epithelial cancer cells (original magnification: 200×). (C) Typical MMP-9 and MMP-2 staining patterns of ovarian carcinomas: two serous carcinomas, one each of mucinous, clear cell, and endometrioid tumors are shown. Adjacent sections from examples of ovarian carcinomas were stained with MMP-2, MMP-9, and collagen IV (original magnification: 100×).

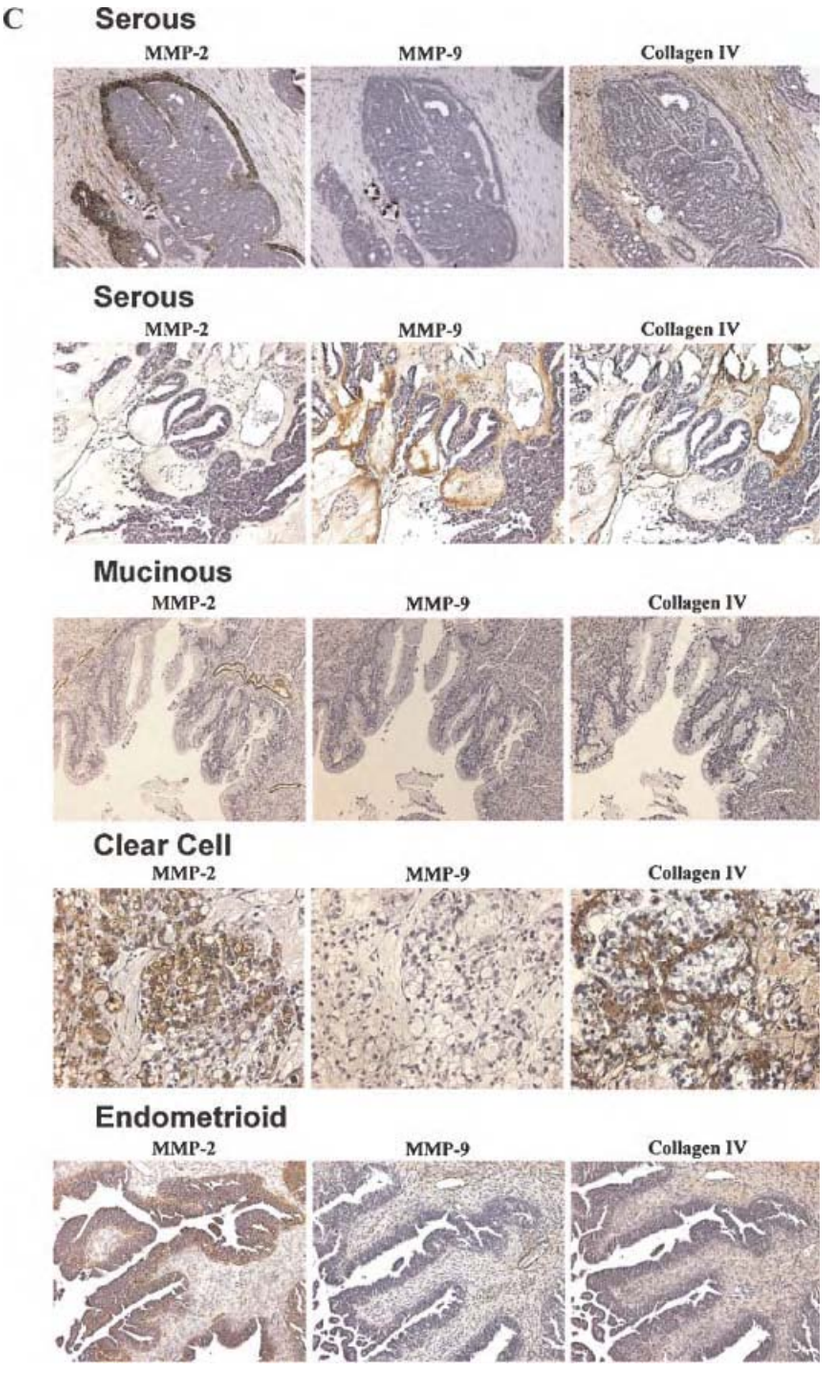

Figure 1. (*Continued*)

Expression of MMP-2 and MMP-9 in Ovarian Tumor Histological Subtypes and Lack of Correlation With Tumor Grades and Stages

Several unique correlations between MMP expression and ovarian tumor histological subtype can be made (Table 2). Mucinous tumors either borderline or malignant often do not express MMPs, because only 1 of the 11 (5 low malignant potential (LMP) and 6 carcinomas) shows expression of MMP-2. This only one MMP-2 positive mucinous tumor is a poorly differentiated carcinoma (see Figure 4A). Most (4 out of 5) undifferentiated ovarian carcinomas lack expression of MMP-2 or MMP-9. Also, 4 out

Table 2. MMP-2 and MMP-9 Immunostaining in Ovarian Tumor Histologic Subtypes

		MMP-2 positive (%)			MMP-9 positive (%)		
Histol. Subtypes	Cases #	EC	S	Total	EC	S	Total
Benign lesions							
Cystadenoma	2	1	0	1 (50%)	0	0	0 (0%)
Borderline							
Serous LMP	8	8	5	8 (100%)	0	5	5 (83.3%)
Mucinous LMP	5	0	0	0 (0%)	0	0	0 (0%)
Malignant							
Serous	65	22	20	33 (50.8%)	0	27	27 (41.5%)
Mucinous	6	1	0	1 (16.7%)	0	0	0 (0%)
Endometrioid	5	4	1	4 (80%)	0	1	1 (33.3%)
Clear Cell	6	4	0	4 (66.7%)	0	0	0 (0%)
Undifferentiated	5	0	0	0 (0%)	0	1	1 (20%)
Metastatic	3	2	2	3 (100%)	0	1	1 (33.3%)
Mal. Mix. Meso.	1	0	0	0 (0%)	0	0	0 (0%)

The data from ovarian tumor blocks and TMA were combined for this analysis.
Abbreviations: EC, epithelial/cancer cells; S, stromal; LMP, low malignant potential; Mal. Mix. Meso., malignant mixed mesodermal.

of 5 endometrioid and 4 of 6 clear cell carcinomas show strong staining of MMP-2 but no MMP-9 in tumor cells (Table 2, Figure 1C). Clear cell carcinomas often stained strongly with collagen IV in the stromal area, as shown in an example in Figure 1C.

Pathological information on the tumors in the TMAs was available to us and we determined the potential correlation between MMP-2 and MMP-9 expression with tumor grades and stages (Table 3). Unexpectedly, we did not find a correlation between MMP expression and tumor grades or stages: the *P*-values are greater than 0.05 indicating the values are not statistically different. The percentages of grade 2 and grade 3 tumors expressing MMP-2 and MMP-9 are not notably different. Also, no progressive increase in MMPs expression that associates with tumor stages was found (Table 3). Additionally, in all eight (two from TMA and six from tumor blocks) serous LMP tumors, strong cytoplasmic staining of MMP-2 was found in tumor cells and/or adjacent stroma (Figure 2A). In five of these serous LMP tumors, high expression of MMP-9 was also found in stroma as shown by a representative example (Figure 2D). Strong cytoplasmic MMP-2 staining was also found in epithelial cells of the benign serous cystadenoma (Figure 3A). Thus, MMP-2 or MMP-9 is prominently expressed in the early stages of tumor development and in less malignant ovarian tumors.

Table 3. Correlation Between MMP-2 and MMP-9 Immunoreaction and Tumor Grades/Stages

		MMP-2 positive (%)			MMP-9 positive (%)		
	Total #	EC	S	Total	EC	S	Total
Tumor grade							
1	2	0	0	0 (0%)	0	1	1 (50%)
2	12	4	0	4 (33.3%)	0	4	4 (33.3%)
3	49	13	15	24 (49.0%)	0	17	17 (34.7%)
LMP	13	8	5	8 (61.5%)	0	5	0 (38.5%)
FIGO stage							
I	9	4	0	4 (44.4%)	0	3	3 (33.3%)
II	2	0	1	1 (50%)	0	1	1 (50%)
III	45	12	12	20 (44.4%)	0	15	15 (33.3%)
IV	11	3	1	4 (36.4%)	0	3	3 (27.3%)

Most of the data was derived from in the three ovarian tissue microarrays in which the information on grades and stages are available. The LMP information includes both tumor blocks and tissue microarray. The one case of benign serous cystadenoma was excluded from this analysis.
Abbreviations: EC, epithelial cancer cells; S, stromal.

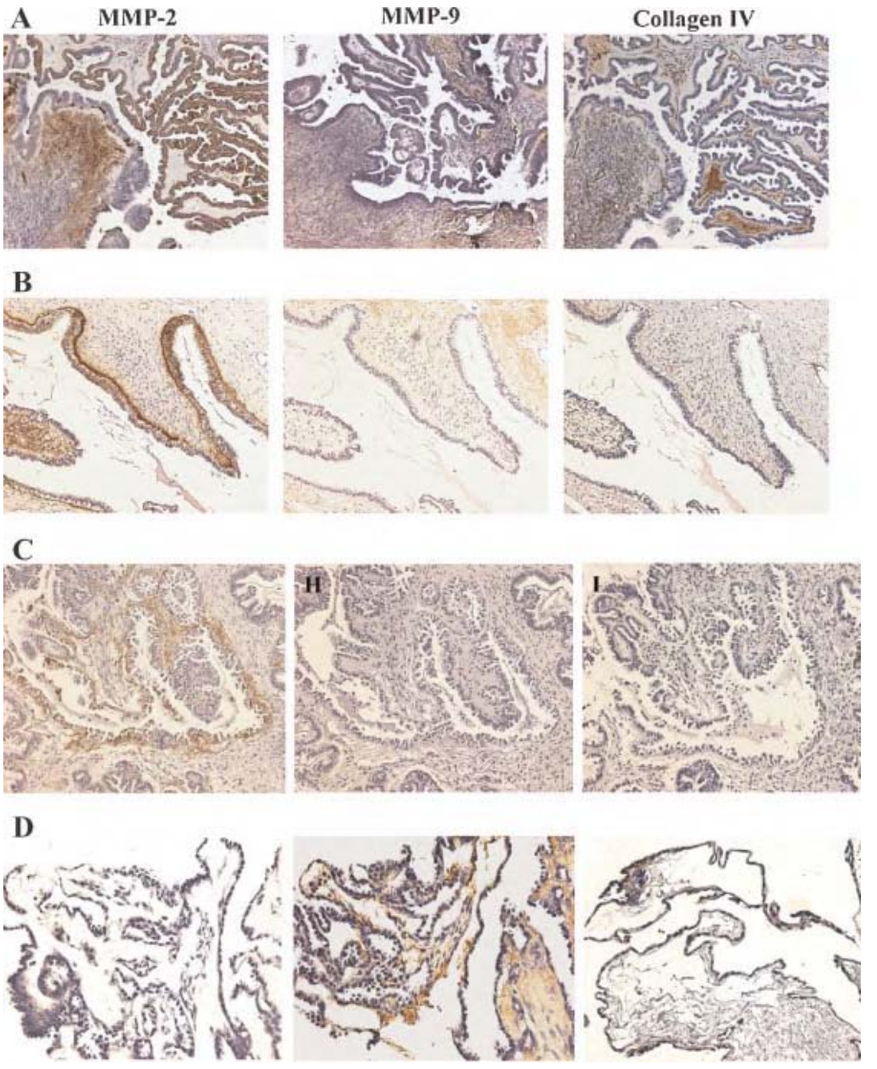

Figure 2. Staining of MMP-2, MMP-9, and collagen IV in LMP. (A) A serous LMP tumor. (B) Both tumor cells and adjacent stroma are positive for MMP-2. (C) An MMP-2 positive, MMP-9 negative LMP tumor. (D) An MMP-2 negative and MMP-9 positive LMP tumor (original magnification: 100×).

Expression of MMP-2 and MMP-9 in Morphological Altered Lesions From Prophylactic Oophorectomies

Prophylactic oophorectomy is often used as a preventive approach for breast and ovarian cancer in a high-risk population [21]. Presumably the ovaries have a higher chance to develop ovarian cancer and contain potential preneoplastic lesions [12]. Here, we examined the expression of MMP-2, MMP-9, and collagen IV by immunostaining in 20 ovarian tissues from prophylactic oophorectomies. In pseudostratification of ovarian surface epithelia, it was consistently observed that MMP-2 instead of MMP-9 is expressed as shown by examples (Figure 3B). In a typical example of papillary lesion shown (Figure 3C), positive MMP-9 staining at the interface between epithelial and stromal compartment correlates with absence of the basement membrane stained by either collagen IV or PAS (arrow), while the areas that lack MMP-9 exhibit basement membrane staining of either collagen IV or PAS (arrowhead).

The results suggest that MMP-2 and MMP-9 are often expressed in areas associated with preneoplastic morphological changes in prophylactic oophorectomies. Also, expression of either MMP-2 or MMP-9 likely accounts for the loss of basement membrane in preneoplastic ovarian surface epithelia.

Expression of MMP-2 and MMP-9 Correlates With the Absence of Basement Membrane

The immunostaining may detect both active or precursor (pro-MMP) forms of MMP-2 and MMP-9. The presence/absence of substrates such as collagen

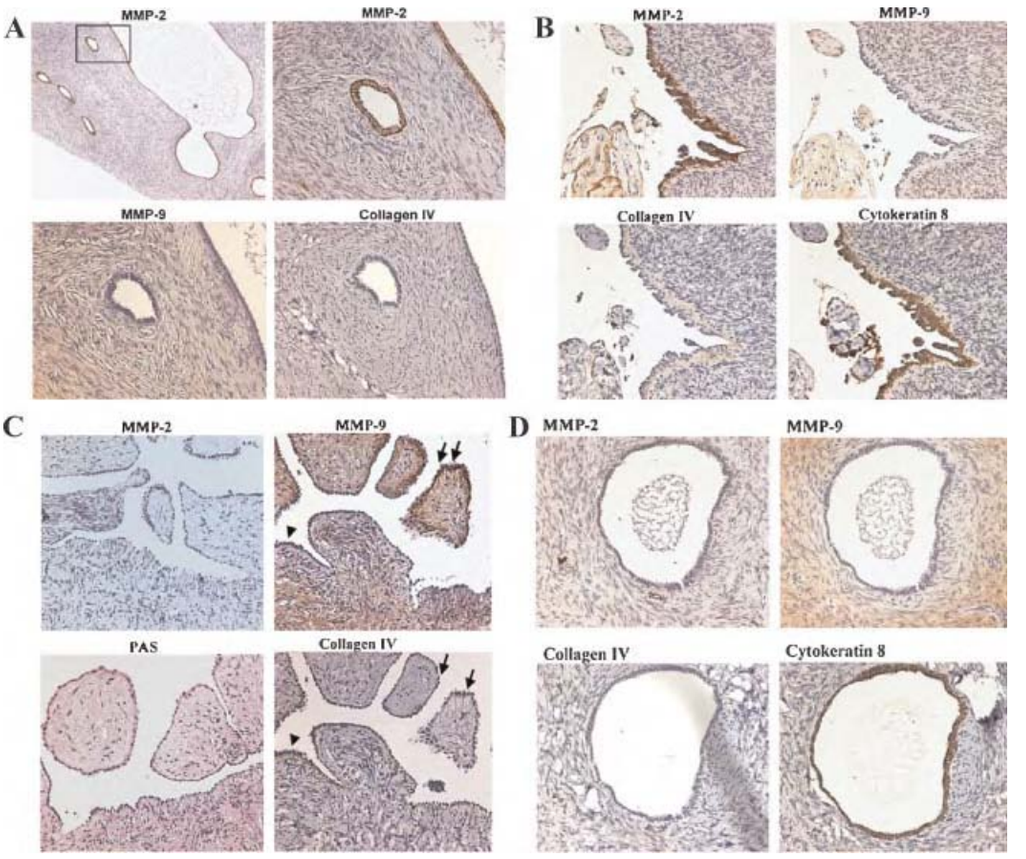

Figure 3. Representative immunostaining of prophylactic oophorectomy tissues. (A) Strong cytoplasmic MMP-2 staining was also found in tumor cells of the benign serous cystadenoma (original magnification: 40×). Higher magnification of the area indicated by an outline in are shown for staining with MMP-2, MMP-9, and collagen IV (original magnification: 200×). (B) The ovarian surface epithelial cells, stained with cytokeratin 8, are hyperplastic and form papillomatosis. MMP-2 is strongly stained (original magnification: 200×). (C) MMP-2 is negative and MMP-9 was detected in the detached papillary structures (original magnification: 200×). The arrows and arrowheads indicate the inverse relationship between the expression of MMP-9 and the presence of basement membrane in corresponding areas of adjacent slides. (D) Lack of MMP-2 and MMP-9 expression in some ovarian cortical inclusion cysts. In some inclusion cysts, no MMP-2 or MMP-9 expression was found, but collagen IV staining was negative. Cytokeratin 8 staining indicates epithelial cells (original magnification: 200×).

IV or laminin in the same area may be used as an indication of the activity of the MMPs. PAS stains can be used to determine if the bulk of basement membrane proteins are present. The expression of either MMP-2 or MMP-9 correlates well with the absence of collagen IV-containing or PAS-stained basement membrane materials in the same areas in prophylactic oophorectomies and most tumor tissues, suggesting the immunoreactive MMP-2 and MMP-9 proteins are active enzymes.

In some inclusion cyst lesions, although collagen IV and PAS staining was absent, indicating the loss of an intact basement membrane, no MMP-2 or MMP-9 expression of was found (Figure 3D). We postulate at least two possibilities. First, proteinases that are capable of degrading the basement membrane, such as uPA and MMP members other than MMP-2 and MMP-9, may be expressed in these lesions. Alternatively, the epithelial cells in the lesions may lose the ability to express, secrete, or assemble basement membrane. The observation that some preneoplastic ovarian epithelial cells and cancer cells do not express collagen IV and/or laminin [5] supports this possibility.

Increased Expression of MMP-9 and MMP-2 in Areas of Histological Transition From Benign to Neoplastic Epithelia in Ovarian Tumors

Even in high-grade cases of ovarian cancer, some benign ovarian tissues attached to tumors may be found occasionally. In these tissues consisting of both benign and tumor portions, contiguous epithelia linking morphological normal to neoplastic lesions can be observed [14–16]. We reason that analysis of markers in these epithelial transition zones may reveal the changing expression from benign to malignant, and may provide clues to the development of ovarian cancer. The expression of

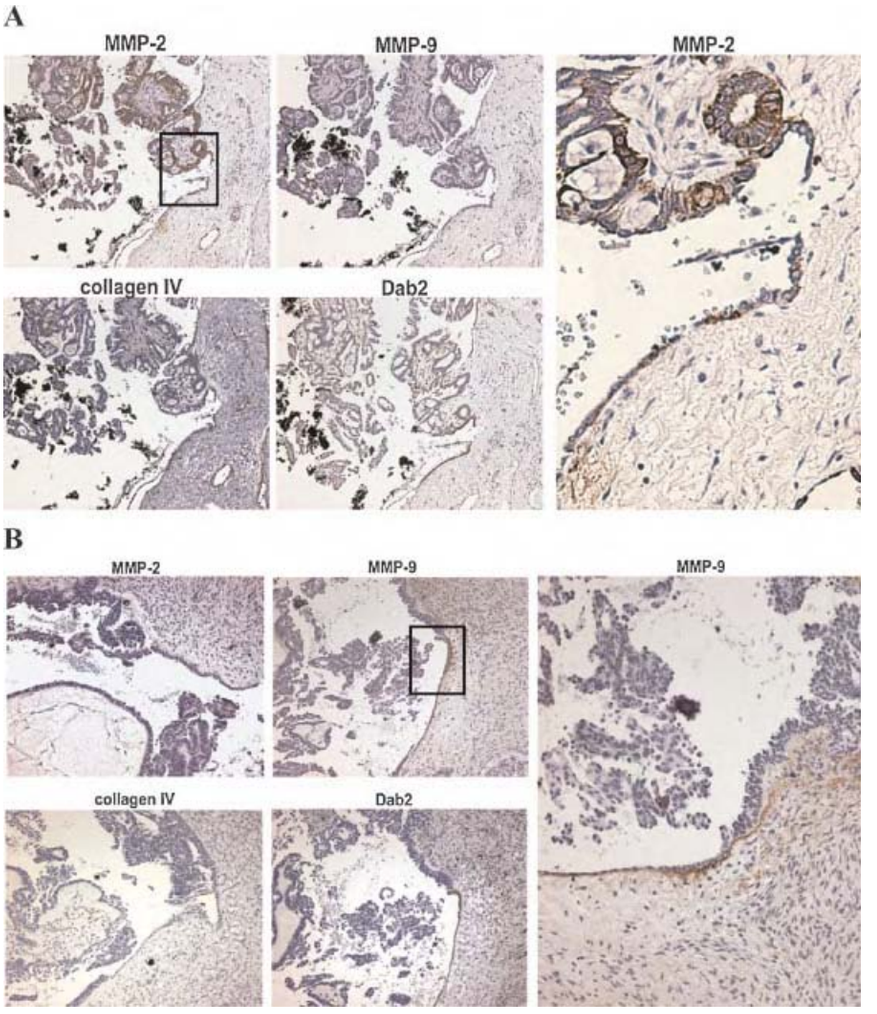

Figure 4. Increased MMP-2 or MMP-9 expression in areas of epithelial morphological transformation. (A) An epithelial transition found in a poorly differentiated mucinous tumor shows MMP-2 positive staining (original magnification: 100×). The boxed area is shown in a higher magnification at the right (original magnification: 200×). (B) In a serous tumor, MMP-9 staining is strong in the transition zone. The area outlined is shown at a higher magnification at the right.

one marker, the putative ovarian cancer tumor suppressor Dab2, correlates well with morphological transition: Dab2 is positive in monolayer but lost in neoplastic, multiple layer lesions [14,15].

We have collected and used 10 ovarian cancer cases containing areas of continuum from normal to neoplastic ovarian epithelia for the current study. We found striking patterns of increased MMP-2 or MMP-9 expression in the areas of epithelial morphological transition. As an example of a case of poorly differentiated mucinous carcinoma, strong cytoplasmic staining of MMP-2 in tumor cells was found in area of transition from benign to malignant epithelium (Figure 4A). Loss of collagen IV and Dab2 was also found in the epithelial transition zone. No MMP-9 expression was observed in this case. As shown in another representative example of a serous carcinoma, the epithelial transition is marked by the loss of Dab2 staining (Figure 4B). MMP-9 staining is strong in the transition zone where the basement membrane (stained by collagen IV) is absent. Such staining pattern of MMP-9 in epithelial transition zones was seen in another three ovarian tumor cases.

In summary, we observed that strong expression of MMP-2 or MMP-9 is associated with the transition of ovarian surface epithelia from morphological normal to neoplastic, suggesting a role for MMP-2 and MMP-9 in epithelial morphological transformation.

Expression of MMPs in Ovarian Epithelial and Cancer Cells

The finding that MMP-2 and MMP-9 expression are more frequent and prevalent in preneoplastic lesions and early stages of ovarian carcinogenesis

than in malignant cancer is somewhat unexpected. Thus, we investigated the expression of MMP-2 and MMP-9 in a panel of nontumorigenic ovarian surface epithelial and cancer cells to further determine the correlation between MMP expression and malignancy of the cells. The several lines of ''HIO'' cells were prepared from morphologically normal ovarian tissues from prophylactic oophorectomy surgeries, and these cells were transfected with SV-40 antigen to prolong the lifespan of the cells in culture [20,22].

Two MMP activities from the condition medium derived from the panel of cells were detected by gelatin zymography (Figure 5A). Based on molecular weights of the MMP enzymes, the activities were likely the latent and active forms of MMP-2 (72 and 62 kDa) and MMP-9 (92 and 82 kDa), respectively. No significant MMP activity was detected in whole cell lysates from any of the cell lines (not shown). The MMP-2 activity is more prominent in the four HIO cell lines than in the panel of ovarian cancer cells (Figure 5A, upper panel), though MMP-2 activity was detected in all cells upon longer development of the zymogram (Figure 5A, lower panel). The presence of the MMP-2 proteins in human ''immortalized'' ovarian surface epithelial cells (HIO cells) was confirmed by Western blot analysis (Figure 5B), but MMP-9 protein was not detectable with Western blot (not shown). It appears that zymography is much more sensitive than immunoblotting to detect both MMP-2 and MMP-9.

Several identical blots of RNA from a panel of HIO and ovarian cancer lines were prepared and several available MMP cDNAs were used to probe their mRNA levels by Northern blot. MMP-1, MMP-3, MMP-9, and MMP-14 were not detectable in both HIO and cancer cells (not shown). MMP-2 mRNA was detected in several lines of HIO cells, but the signal was absent or very low in most cancer cells (Figure 5C). As an exception, ES2 ovarian cancer cells express a high level of MMP-2 (Figure 5C). ES2 is an ovarian clear cell carcinoma line, thus, it is consistent with our observation that MMP-2 is often expressed in ovarian clear cell carcinomas. We noticed that MMP-2 expression pattern exhibits some correlation with the expression of the differentiation-determining transcription factor GATA6 (Figure 5C). For example, MMP-2 and GATA6 are both expressed in HIO-103, HIO-115, HIO-114, ES2, and SKOV-3 cells, but not (or weak) in A2780, OVCAR-2, -3, -4, -5, -8, and -10 cells. In HIO-118 and HIO-107 cells, however, the expression of MMP-2 and GATA6 are not correlative well.

Based on the data from zymography and results from Western and Northern blots, it appears that cancer cells generally express little or no MMPs mRNA and proteins compared to the

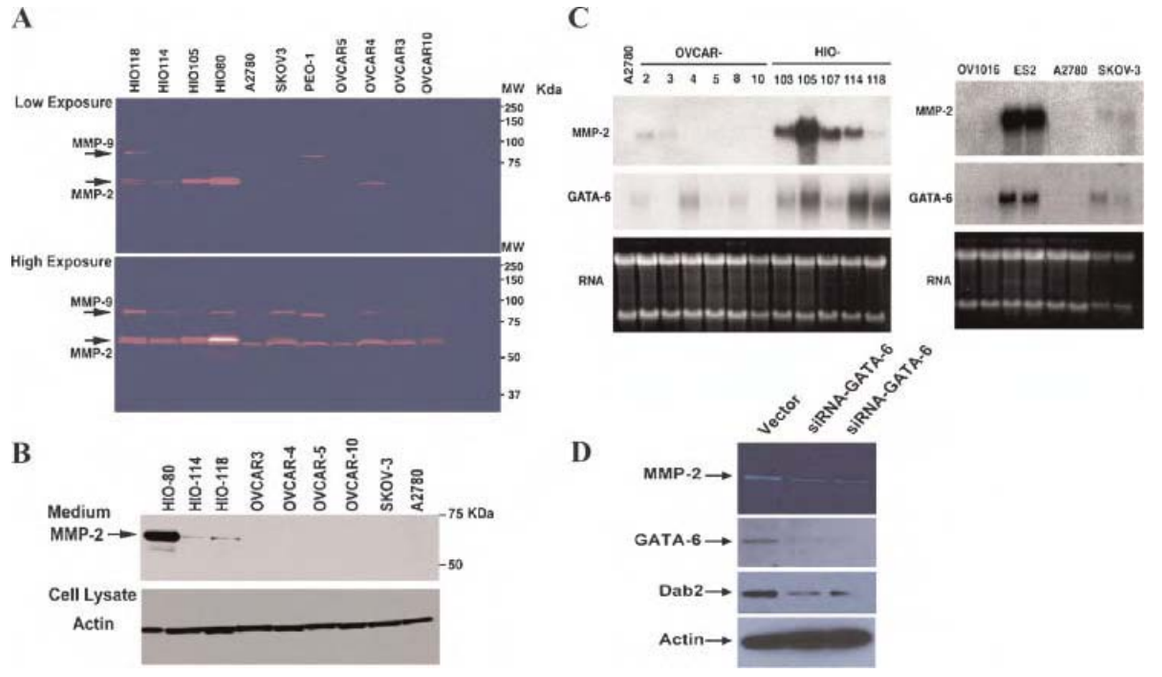

Figure 5. Expression of MMPs in ovarian surface epithelial and cancer cell lines. (A) MMP-2 and MMP-9 activities were detected by zymography. (B) The condition medium was analyzed for MMP by Western blot. The amount of cells, from which the condition medium was obtained, were determined by Western blotting analysis of the cell lysate for β-actin level. (C) Total RNA was extracted from a panel of ovarian epithelial and cancer cells for analysis by Northern blotting. (D) ES2 cells were transfected with either vector as control or siRNA targeting the downregulation of GATA6. Two separate flasks of cells transfected with siRNA were included. The next day, condition medium was collected for a 4-h period. MMP-2 activity was detected by zymography in the condition medium, and the cells were harvested for Western blot analysis of GATA6, Dab2, and β-actin. This experiment was repeated three times with similar result.

nontumorigenic cells. The findings from the cell lines are consistent with the conclusion derived from tissue analysis that MMPs, especially MMP-2, is more frequently expressed in nontumorigenic cells and preneoplastic lesions than in malignant cancer cells.

Regulation of MMP Expression in Ovarian Epithelial and Cancer Cells

We tested the hypothesis that GATA6 may regulate MMP-2 expression by downregulation of GATA6 expression in ES2 cells (Figure 5D). Western blotting of the cell lysate showed that GATA6 was downregulated by the siRNA transfection and the expression of *Dab2*, a GATA6-dependent gene, was also reduced. MMP-2 activity in the condition medium detected by zymography was also reduced to 30% of the vector control (Figure 5D). Thus, the result supports that GATA6 may regulate MMP-2 expression, and the reduced MMP-2 in ovarian cancer cells may due to the loss of GATA6 expression in ovarian cancer [20].

Because MMP-9 expression in ovarian surface epithelial cells is regulated by TNF-α [9], we also investigated the regulation in ovarian cancer cells. In contrast to HIO cells [9], in two independent preparations of primary human ovarian surface epithelial cells tested, TNF-α failed to stimulate MMP-9 expression (Figure 6A). In a panel of HIO and ovarian cancer cells tested (Figure 6B), we found that MMP-9 is expressed and stimulated by TNF-α in HIO-80, SKOV-3 (weakly), Peo-1, and UPN cells, but not in HIO-118, OVCAR3, OVCAR5, OVCAR10, ES2, A1847 cells. Thus, only a fraction of ovarian cancer cells express MMP-9 and are responsive to TNF-α.

DISCUSSION

Previously we found that preneoplastic ovarian surface epithelia often lack intact basement membranes and proposed that loss of basement membrane is a step in neoplastic morphological transformation [5]. In the current study we set out to determine if expression of metalloproteinases may be the cause of the loss of basement membrane. We systematically investigated MMP-2 and MMP-9 expression in preneoplastic ovarian tissues, tumors, and cultured cells. The presence of a basement membrane was also determined by PAS and collagen IV staining. The current results indicate that the increased expression of MMP-2 and MMP-9 is correlative and may be responsible for the loss of ovarian epithelial basement membrane in most cases. The study also reaches an unexpected conclusion that MMP-2 and MMP-9 expression is more prominent in preneoplastic lesions and early stages of tumor development than in neoplastic cells and overt malignant cancers. A role for MMP expression

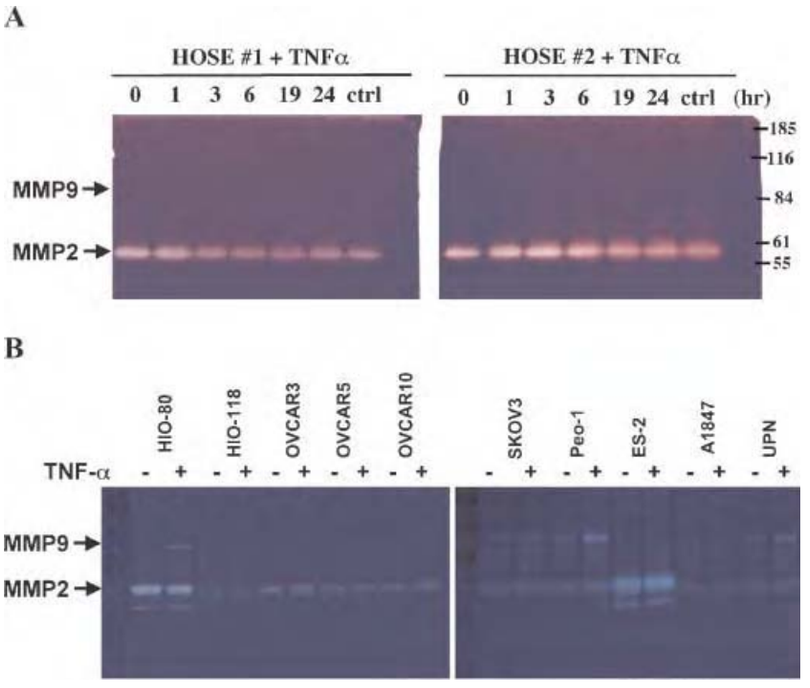

Figure 6. Regulation of MMPs in ovarian surface epithelial and cancer cell lines by TNF-α. (A) Two independent preparations of primary human ovarian surface epithelial (HOSE) cells were stimulated with TNF-α (10 ng/mL). Condition medium was collected at the indicated time following TNF-α stimulation and used for zymography to determine MMP activities. (B) HIO and ovarian cancer cells were stimulated with or without TNF-α (10 ng/mL) and the condition medium was collected after 18 h. The condition medium was analyzed for MMP activities by zymography.

in morphological transformation is also suggested based on the observations of MMP immunostaining in epithelial transitions from benign to malignant areas in tumor tissues.

Regulation of MMPs Expression in Ovarian Epithelial and Cancer Cells

In cultured ovarian surface epithelial cells, MMP-9 expression was shown to be regulated by TNF-α and IL-1β [9]. These pro-inflammatory cytokines are implicated in the rupture of the ovarian surface during ovulation, which is an inflammation-like process [23]. The stimulation of MMP-9 by TNF-α is believed to be part of the gonadotropin-stimulated cascade of events in ovulation, including proteolytic degradation of tissue and basement membrane to allow the release of ova. Furthermore, it was speculated that TNF-α-stimulated MMP-9 expression and tissue remodeling represents an underlying mechanism for the etiological link between ovulation and ovarian cancer risk [9]. Presumably, the increases in MMP-9 expression at the junctions between morphological normal and neoplastic lesions observed in the current study are due to the stimulation of local concentrations of TNF-α and IL-1β. However, it is peculiar that primary ovarian surface epithelial cells are not responsive to TNF-α for the expression of MMP-9. Also, most of the ovarian cancer cells also do not express MMP-9 or responsive to TNF-α. We speculate that ovarian surface epithelial cells need to be activated to become competent for TNF-α-regulated MMP-9 expression. The nature of such activation is unknown.

MMP-2 is known to be regulated both at the transcription level [24,25] and posttranscriptional activation by MT1-MMP and inhibition by an endogenous inhibitor, TIMP-1 [26]. MMP-2 expression can be modulated by growth factors through the Ras/MAPK and AKT pathways [24,26], though the response is gradual and MMP-2 is not an immediate early response gene. We found that MMP-2 expression is more prominent in benign and early stages of ovarian tumor development than established tumors. Consistently, MMP-2 was found expressed often in the nontumorigenic HIO cells rather than in cancer cells, which was especially obvious in the Northern blot to measure MMP-2 mRNA. A correlation between expression of GATA-6 and MMP-2 is present: expression of both genes are present in most HIO lines and ES2 cells but absent or very low in most other ovarian cancer cells tested. Interestingly, a previous study found that MMP-2 promoter contains a GATA binding site and GATA factors stimulates MMP-2 expression in endothelial cells [25]. Thus, we especially favor a hypothesis that MMP-2 expression is lost in neoplastic ovarian epithelial cells due to the loss of the differentiation-determine GATA transcription factors, a process we defined as epithelial dedifferentiation [20]. The finding in experiments using siRNA to downregulate GATA6 in ES2 cells supports this hypothesis.

Increased Expression of MMPs is an Early Event in Ovarian Tumorigenesis

The roles of MMP expression in tumor invasion and metastasis and the correlation between MMP expression and tumor malignancy are dogmas in tumor biology [27]. For ovarian cancer, secretion of MMP-2 and MMP-9 has been observed in several ovarian cancer cell lines and detected in ascetic fluid from patients with advanced ovarian cancer [28]. MMP expression is prominent in ovarian tumors [29–31], the invasiveness of ovarian cancer cell lines correlates with the expression of MMP-2 and MMP-9 in vitro [32], and correlation of MMP expression with patient prognosis [33]. It is generally believed that MMP expression correlates with tumor progression and the degree of malignancies. Our conclusions that MMP-2 and MMP-9 expression is more prominent in early and preneoplastic lesions than overt ovarian tumors, and a lack of correlation between MMP expression with tumor grades and stages, are quite unique. We attribute our distinct findings and conclusions to the new availability of preneoplastic ovarian tissues and cells for study.

Clinical trials with MMP inhibitors as anti-cancer therapy have yielded disappointing results [34,35]. Further studies led to the suggestion that MMPs have functions other than invasion and metastasis, and that they act before invasion occurs in the development of cancer. Our conclusion is consistent with the new understanding that MMPs play important roles in tumor initiation [34,35].

Expression of MMPs Associates With Ovarian Epithelial Morphological Transformation

An intriguing observation is the increased expression of MMP-2 and MMP-9 in the areas of histological transition from benign to malignant epithelia in ovarian tumor tissues. High expression of MMP-2 and MMP-9 was also identified in the preneoplastic morphological changes in ovaries obtained from prophylactic oophorectomies.

MMPs play a biological role in tissue morphogenesis during development, possibly by regulating the integrity of epithelial basement membrane [2,3,34,36]. Thus, we propose that the increased expression of MMP-2 and MMP-9 is a causative factor in ovarian morphological alterations and they promote neoplastic growth of predisposed (containing genetic or epigenetic changes) epithelial cells to develop tumors.

Increased MMP Expression as an Etiological Factor for Ovarian Cancer

The link between ovulation and ovarian cancer risk has been well recognized [37,38]. The

gonadotropin stimulation hypothesis postulates that the ovulation-associated surges of gonadotropins are the causative factors for the neoplastic transformation of ovarian surface epithelial cells [37,38]. Even after cessation of ovulation in menopausal, the increased gonadotropins stimulate an ovulation-like inflammatory process [39], which was also considered an etiological factor in ovarian cancer risk. Thus, these gonadotropin-stimulated an increase of cytokines such as TNF-α and IL-1β and further induce expression of MMP-9, which subsequently lead to the degradation of ovarian surface epithelial basement membrane and morphological alteration. Ultimately, the increases in MMP-2 and MMP-9 may lead to the selection and promotion of neoplastic cells.

Hence, the increased expression of MMP-2 and MMP-9 in ovaries may be considered an etiological factor in ovarian cancer risk, and a reduction of ovarian MMP activity may be an approach in ovarian cancer prevention.

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REFERENCES

1. Auersperg N, Wong AS, Choi KC, Kang SK, Leung PC. Ovarian surface epithelium: Biology, endocrinology, and pathology. Endocr Rev 2001;22:255–288.
2. Bissell MJ, Barcellos-Hoff MH. The influence of extracellular matrix on gene expression: Is structure the message? J Cell Sci 1987;8:327–343.
3. Adams JC, Watt FM. Regulation of development and differentiation by the extracellular matrix. Development 1993;117:1183–1198.
4. Capo-Chichi CD, Smith ER, Yang DH, Roland IH, Vanderveer L, Cohen C, Hamilton TC, Godwin AK, Xu XX. Dynamic alterations of the extracellular environment of ovarian surface epithelial cells in premalignant transformation, tumorigenicity, and metastasis. Cancer 2002;95:1802–1815.
5. Roland IH, Yang WL, Yang DH, Daly MB, Ozols RF, Hamilton TC, Lynch HT, Godwin AK, Xu XX. Loss of surface and cyst epithelial basement membranes and preneoplastic morphologic changes in prophylactic oophorectomies. Cancer 2003;98:2607–2623.
6. Matrisian LM. The matrix-degrading metalloproteinases. Bioessays 1992;14:455–463.
7. Mott JD, Werb Z. Regulation of matrix biology by matrix metalloproteinases. Curr Opin Cell Biol 2004;16:558–564.
8. Stack MS, Ellerbroek SM, Fishman DA. The role of proteolytic enzymes in the pathology of epithelial ovarian carcinoma. Int J Oncol 1998;12:569–576.
9. Yang WL, Godwin AK, Xu XX. Tumor necrosis factor-alpha-induced matrix proteolytic enzyme production and basement membrane remodeling by human ovarian surface epithelial cells: Molecular basis linking ovulation and cancer risk. Cancer Res 2004;64:1534–1540.
10. Feeley KM, Wells M. Precursor lesions of ovarian epithelial malignancy. Histopathology 2001;38:87–95.
11. Resta L, Russo S, Colucci GA, Prat J. Morphologic precursors of ovarian epithelial tumors. Obstet Gynecol 1993;82:181–186.
12. Salazar H, Godwin AK, Daly MB, et al. Microscopic benign and invasive malignant neoplasms and a cancer-prone phenotype in prophylactic oophorectomies. J Natl Cancer Inst 1996;88:1810–1820.
13. Barakat RR, Federici MG, Saigo PE, Robson ME, Offit K, Boyd J. Absence of premalignant histologic, molecular, or cell biologic alterations in prophylactic oophorectomy specimens from BRCA1 heterozygotes. Cancer 2000;89:383–390.
14. Yang DH, Smith ER, Cohen C, et al. Molecular events associated with dysplastic morphologic transformation and initiation of ovarian tumorigenicity. Cancer 2002;94:2380–2392.
15. Fazili Z, Sun W, Mittelstaedt S, Cohen C, Xu XX. Loss of Disabled-2 is an early step in ovarian tumorigenicity. Oncogene 1999;18:3104–3113.
16. Puls LE, Powell DE, DePriest PD, et al. Transition from benign to malignant epithelium in mucinous and serous ovarian cystadenocarcinoma. Gynecol Oncol 1992;47:53–57.
17. Sheng Z, Sun W, Smith E, Cohen C, Sheng Z, Xu XX. Restoration of positioning control following Disabled-2 expression in ovarian and breast tumor cells. Oncogene 2000;19:4847–4854.
18. Mok SC, Chan WY, Wong KK, et al. *DOC-2*, a candidate tumor suppressor gene in human epithelial ovarian cancer. Oncogene 1998;16:2381–2387.
19. Yang D-H, Smith ER, Roland IH, et al. Disabled-2 is essential for endodermal cell positioning and structure formation during mouse embryogenesis. Dev Biol 2002;251:27–44.
20. Capo-Chichi CD, Roland IH, Vanderveer L, et al. Anomalous expression of epithelial differentiation-determining GATA factors in ovarian tumorigenesis. Cancer Res 2003;63:4967–4977.
21. Haber D. Prophylactic oophorectomy to reduce the risk of ovarian and breast cancer in carriers of BRCA mutations. N Engl J Med 2002;346:1660–1662.
22. Maines-Bandiera SL, Kruk PA, Auersperg N. Simian virus 40-transformed human ovarian surface epithelial cells escape normal growth controls but retain morphogenetic responses to extracellular matrix. Am J Obstet Gynecol 1992;167:729–735.
23. Richards JS, Russell DL, Ochsner S, Espey LL. Ovulation: New dimensions and new regulators of the inflammatory-like response. Annu Rev Physiol 2002;64:69–92.
24. Liao J, Wolfman JC, Wolfman A. K-ras regulates the steady-state expression of matrix metalloproteinase 2 in fibroblasts. J Biol Chem 2003;278:31871–31878.
25. Han X, Boyd PJ, Colgan S, Madri JA, Haas TL. Transcriptional up-regulation of endothelial cell matrix metalloproteinase-2 in response to extracellular cues involves GATA-2. J Biol Chem 2003;278:47785–47791.
26. Corcoran ML, Hewitt RE, Kleiner DE Jr, Stetler-Stevenson WG. MMP-2: Expression, activation and inhibition. Enzyme Protein 1996;49:7–19.
27. Crawford HC, Matrisian LM. Tumor and stromal expression of matrix metalloproteinase and their role in tumor progression. Invasion Metastasis 1994;14:234–245.

28. Moser TL, Young TN, Rodriguez GC, Pizzo SV, Bast RC, Jr., Stack MS. Secretion of extracellular matrix-degrading proteinases is increased in epithelial ovarian carcinoma. Int J Cancer 1994;56:552–559.
29. Huang LW, Garrett AP, Bell DA, Welch WR, Berkowitz RS, Mok SC. Differential expression of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 protein and mRNA in epithelial ovarian tumors. Gynecol Oncol 2000;77: 369–376.
30. Schmalfeldt B, Prechtel D, Harting K, et al. Increased expression of matrix metalloproteinases (MMP)-2, MMP-9, and the urokinase-type plasminogen activator is associated with progression from benign to advanced ovarian cancer. Clin Cancer Res 2001;7:2396–2404.
31. Garzetti GG, Ciavattini A, Lucarini G, et al. Expression of vascular endothelial growth factor related to 72-kilodalton metalloproteinase immunostaining in patients with serous ovarian tumors. Cancer 1999;85:2219–2225.
32. Naylor MS, Stamp GW, Davies BD, Balkwill FR. Expression and activity of MMPS and their regulators in ovarian cancer. Int J Cancer 1994;58:50–56.
33. Lengyel E, Schmalfeldt B, Konik E, et al. Expression of latent matrix metalloproteinase 9 (MMP-9) predicts survival in advanced ovarian cancer. Gynecol Oncol 2001;82:291–298.
34. Egeblad M, Werb Z. New functions for the matrix metalloproteinases in cancer progression. Nat Rev Cancer 2002;2: 161–174.
35. Coussens LM, Fingleton B, Matrisian LM. Matrix metalloproteinase inhibitors and cancer: Trials and tribulations. Science 2002;295:2387–2392.
36. Rudolph-Owen LA, Chan R, Muller WJ, Matrisian LM. The matrix metalloproteinase matrilysin influences early-stage mammary tumorigenesis. Cancer Res 1998;58:5500–5506.
37. Fathalla MF. Incessant ovulation—A factor in ovarian neoplasia? Lancet 1971: 2:163.
38. Cramer DW, Welch WR. Determinants of ovarian cancer risk. II. Inferences regarding pathogenesis. J Natl Cancer Inst 1983;71:717–721.
39. Smith ER, Xu XX. Etiology of epithelial ovarian cancer: A cellular mechanism for the role of gonadotropins. Gyn Oncol 2003;91:1–2.